PROCEEDINGS OF THE THIRD SYMPOSIUM ON REPRODUCTION AND INFERTILITY

REPRODUCTION AND INFERTILITY

III SYMPOSIUM

COLORADO STATE UNIVERSITY
Fort Collins Colorado

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Sponsored by
The College of Veterinary Medicine
and
The Agricultural Experiment Station

SYMPOSIUM PUBLICATIONS DIVISION
PERGAMON PRESS
LONDON NEW YORK PARIS LOS ANGELES
1958

PERGAMON PRESS LTD
4 & 5 Fitzroy Square London W 1

PERGAMON PRESS INC
122 East 55th Street New York 22 N Y
P O Box 47715 Los Angeles California

PERGAMON PRESS S A R L
*24 Rue des Écoles Paris V^e^*

Library of Congress Card No 58–13134

Print d in G e t B itai by
Robert MacLeh e and Company Limit d
The Uni ity Pr s Gla o w

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PREFACE

THE creation of a biennial Symposium on Reproduction and Infertility was the result of widespread demands by investigators and specialists active in the animal and human fields Unquestionably there is an urgent need for a greater opportunity to hold fruitful discourse on the many complex problems confronting the researcher in the study of reproduction infertility and disease and for offering a means of effectively emphasizing the cosmopolitan and limitless nature of the problems attending reproduction

As the literature pertinent to these and many new phases is widely scattered and often known only to specialists it was thought that a useful purpose would be served if this newer knowledge born of basic research, could be aired and disseminated through these symposia to interested students teachers and researchers

The first Symposium on Reproduction and Infertility was held at Iowa State College in 1953 This relatively small but successful gathering was followed by Symposium II at Michigan State University during its Centennial celebration in 1955 Colorado State University was host to Symposium III on Reproduction and Infertility on July 1–4 1957 That these meetings are held in high regard by investigators and clinicians active in these fields is indicated by the capacity attendance which showed a unique cosmopolitan character representing thirty eight states and eight foreign countries Considerable effort was made to record and transcribe the pertinent discussions particularly those held during Panel Sessions an innovation in the publication of these symposia

In the selection of papers for presentation before the Symposium preference was given to those topics in which advance has been rapid in recent years and which would best serve to cover the more important facets of the problems encountered in reproduction and infertility Each author was given generous latitude in organizing and defending his views in his own area or field of specialization as this serves to stimulate and guide research towards the crystallization of theories and the estabishment of truth

F X GASSNER
Editor

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Acknowledgments

Colorado State University gratefully acknowledges the co-sponsorship of the following pharmaceutical organizations participating in this Symposium

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THE PROBLEM OF WORLD WIDE RADIOACTIVE FALLOUT FROM NUCLEAR WEAPONS TESTING

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THE problem of world wide radioactive fallout from weapons test operations is one of the most controversial issues brought to the public s attention in many years It was made an issue in the recent presidential election It is a subject frequenting the front pages of many newspapers and is an important subject of discussion at current disarmament conferences Only last month the potential hazard of world wide radioactive fallout was the subject of a Con gressional subcommittee hearing

Consideration of the potential hazard from world wide radioactive fallout may start by calling attention to three important facts

(1) There is no doubt but that the world population is receiving a small ex posure to radioactive materials originating from nuclear weapons testing Fission products from bomb detonations have been and are depositing over the surface of the earth increasing the external gamma radiation background and finding their way into the human body through transmission along the eco logical cycle from soil to-plants to-animals and to man

(2) It is a well-established fact that *enough radiation* either from an external source or from radioactive isotopes deposited in the body will produce de leterious effects These effects may result in an increase in genetic mutations shortening of life expectancy and increased incidence of leukemia and bone sarcoma

(3) Radiation exposure is not a new experience for the world population All living things have been exposed to radiation since the beginning Radiation from cosmic rays from radioactive minerals in the earth s crust and from radium potassium-40 carbon 14 and thorium deposited in the body consti tute this so-called natural background The amount of natural background radiation is such that the average person who lives to an age of 70 years re ceives a total of about 7 roentgens while his skeleton (as a result of radium and other radioactive materials deposited in his bones) receives the equivalent of 10–12 roentgens

Of greatest concern are the genetic effects of radiation to the gonads and the possible leukemogenic and sarcogenic effects of Sr^{90} deposited in the bones

This report is confined to consideration of the Sr^{90} aspect of the fallout problem is the one with which this author is most familiar

Based on the above basic facts, the purpose of this report is to summarize as factually as possible from existing data the potential hazard of Sr^{90} from nuclear weapons testing

GENERAL DISTRIBUTION OF WORLD WIDE FALLOUT

Based on soil measurements Dr W F Libby of the AEC has proposed a mechanism by which atomic debris is disseminated throughout the world This theory postulates three kinds of fallout local tropospheric and strato spheric

The first type, local fallout is deposited in the immediate environs of the explosion during the first few hours This debris consists of the large particles from the fireball and includes partially or completely vaporized residues from the soil and structures which are swept into the cloud The fraction of total radioactivity which falls out locally is largely dependent on those firing condi tions which govern the amounts of soil and extraneous debris incorporated in the fireball Local fallout however is of little concern since it is confined to uninhabited areas and does not get widely disseminated in the biosphere

The second type tropospheric fallout consists of that material injected into the atmosphere below the tropopause which is not coarse enough to fall out locally *This debris is sufficiently fine that it travels great distances circling* the earth in the general latitude of the explosion until removed from the atmo sphere by rain fog contact with vegetation and other meteorological and/or *physical factors The average tropospheric fallout time is estimated at* 20–30 days The fraction of fallout in this category depends mainly on the size of the explosion and conditions of firing If the explosion exceeds a certain minimum size (about one megaton i e equivalent in energy released to 1 million tons of TNT) the fireball will have enough energy to penetrate the tropopause carrying fission products into the stratosphere Smaller detona tions leave in the troposphere all debris not deposited locally The fraction of the fission products from a large weapon that remains in the tropopause de pends on the size of the explosion conditions of firing and meteorological factors

The third type stratospheric fallout is composed of fission products carried above the tropopause and can result only from large weapons These are be lieved to mix rapidly throughout the stratosphere and fall back uniformly into the troposphere where they are deposited over the earth s surface in relation to meteorological conditions The average deposition time of stratospheric debris is estimated at from 6 to 10 years

The above mechanism *leads to a general distribution of radioactivity over*

the surface of the earth with a higher concentration in the north temperate latitudes Estimates of Sr^{90} levels in the fall of 1956 suggest 22 mc/mile² for the northern sections of the United States 15–17 mc/mile² for similar latitudes elsewhere in the world and 3–4 mc/mile² for the rest of the world Actually this general picture is greatly oversimplified Once fission products are sus pended in the troposphere (either directly by the detonation or by air exchange *regardless of mechanism between the troposphere and the stratosphere)* meteorological conditions play a major role in their deposition Within any major fallout area one might expect to find fluctuations in the level of surface deposition which correlate with local meteorological conditions

Even if no more bomb tests were held surface deposition levels will con tinue to increase until about 1970 because of fallout of fission products still in the stratosphere It is estimated that the stratospheric reservoir (in the fall *of 1956) contained the products of about 24 megatons of fission One megaton* of fission results in the formation of enough Sr^{90} to give a surface deposition of 0 5 mc/mile² if uniformly distributed over the entire surface of the earth If all material presently in the stratospheric reservoir were deposited instan taneously and uniformly over the earth present values would be increased by 12 mc/mile² and the maximum surface deposition of Sr^{90} would result Maxi mum deposition however will not occur because of the relatively long average *stratospheric storage time (6–10 years) which will allow some of the stron* tium to decay before deposition

Under these conditions the area in the northern United States would be expected to reach a level of about 29 mc/mile² The area between 60 –10 N latitude may reach about 23 mc/mile² and the rest of the world may reach a level of about 10 mc/mile² These values are general levels only assuming uniform distribution within the respective areas Local meteorological conditions will *produce nonuniformities within these general regions Recent data suggest* that some areas of the United States (South Dakota Iowa Michigan New York) already may have deposition levels of about 30 mc/mile² (January 1957)

INCORPORATION OF STRONTIUM 90 INTO THE BIOSPHERE AND INTO MAN

When Sr^{90} falls upon the earth s surface it is taken into plants through the root system in relation to the available calcium in the soil That which settles directly on vegetation may remain as surface contamination or a part of it may enter the plant through foliate absorption When plants are eaten by animals Sr^{90} deposited directly on the surface or incorporated in the plant (by foliate absorption or from the soil) is absorbed by the animal along with calcium When plant and animal products (e g milk) are eaten by man the Sr^{90} they contain becomes incorporated with his body calcium and deposits predominantly in the bone

It is reasonable to assume that strontium may be discriminated against with respect to calcium in passing along the ecological chain For example the Sr^{90}/Ca ratio in human bones may be expected to be lower than the Sr^{90}/Ca ratio in the soil where the ecological chain between man and his environment begins

Attempts are being made (using radioactive tracers) to determine the over all Sr^{90}/Ca discrimination ratio in going from soils to human bone by indi vidually determining the discrimination factors (DF) that occur at the various steps along the ecological chain

The discrimination factor most difficult to establish is that from soils to plants (DF_1) It is dependent among other things on type of soil available soil calcium type of plant and perhaps on rainfall all of which may vary greatly with geographic location The soil to plant discrimination factor has been estimated as about 0 7 i e $\frac{(Sr^{90}/Ca)_{plant}}{(Sr^{90}/Ca)_{soil}} = 0\ 7$

The discrimination factor from plants to milk (DF) has been estimated as about 0 13 and the discrimination factor (DF_3) in going from milk to human bone equals about 0 5 Experiments on the discrimination factor (DF_4) from plants directly to human bone give a value of about 0 25

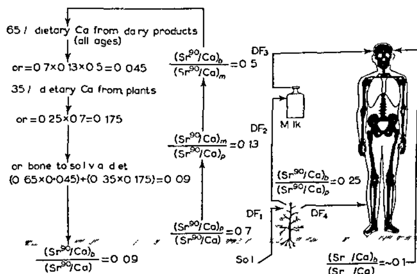


FIG 1 Discrimination against strontium with respect to calcium in passing up the food cycle from soil to man

The over all ratio (OR $_{bone-soil}$) in going from soil to human bone via the diet may be estimated from the various discrimination factors and the fraction of dietary calcium derived from dairy products and directly from other sources The Department of Agriculture on the basis of United States retail sales has

estimated that 65% of the dietary calcium for all ages comes from dairy pro ducts and 35% from other sources On this basis

$$OR = (0\,65 \times DF_1 \times DF_2 \times DF_3) + (0\,35 \times DF_1 \times DF_4)$$
$$= (0\,65 \times 0\,7 \times 0\,13 \times 0\,5) + (0\,35 \times 0\,7 \times 0\,25) = 0\,09$$

This value indicates that the Sr^{90} concentration in the bone calcium will be only about 0 1 that in the available soil calcium

The above information on the over all discrimination factor against stron tium during passage up the ecological chain from soils to human bone via the diet is summarized in FIG 1 and may be used to estimate the average present and future maximum Sr^{90} levels in bone as a result of weapons tests to date In so doing however it must be emphasized that these values are for *ecological* discrimination and apply only to passage along the ecological chain An ecological discrimination factor automatically assumes that the calcium and strontium are uniformly mixed in soil and all of the Sr^{90} is in an available form to the depth of the plant feeding zone No allowance is made for direct foliar absorption of Sr^{90} for its dilution with a greater reservoir of available soil calcium through plowing or for the possibility that it may become less avail able with time through soil binding and leaching

PREDICTED PRESENT AND FUTURE STRONTIUM 90 AVERAGE MAXIMUM LEVELS IN BONE

Sr^{90} content of the bones of the population when in equilibrium with time of maximum biospheric contamination is the major concern From the present and predicted maximum Sr^{90} surface deposition levels and the soil to bone discrimination ratios derived in the previous section present and future average maximum Sr^{90} levels in bone can be predicted

Assuming an average of 20 g available Ca/ft² of soil to a depth of 2½ in 1 mc of Sr^{90}/mile is equivalent to 1 8 $\mu\mu$c Sr^{90}/g available soil calcium Multipli cation of the Sr^{90} surface deposition levels given earlier by 1 8 gives the Sr^{90} activity per gram of available soil calcium Multiplication of the specific activity of the available soil calcium by the Sr^{90} discrimination ratio of 0 09 should give the average maximum specific activity of calcium l id down in the adult skeleton through exchange and bone remodeling during the period of environmental contamination and the maximum Sr^{90} concentration in the bones of children who may be approaching an equilibrium state

Such calculations suggest that the average maximum equilibrium level as of the fall of 1956 should be about 3 6 2 6 and 1 3 $\mu\mu$c Sr^{90}/g Ca for the northern United States the area between 60 –10 N latitude and the world average respectively Sr^{90} analyses of bone samples from all ages collected from all over the world actually show a much lower concentration than the predicted values Children s bones average about 0 5–0 9 $\mu\mu$c/g Ca while adult bones average only about 0 1 $\mu\mu$c/g Ca Part of this discrepancy is due to the fact that the

bone samples do not represent equilibrium conditions since much of the skeleton (especially of adults) was formed before fallout from bomb tests started When the bone analyses are converted to equilibrium conditions they suggest that equilibrium levels in the fall of 1956 should be about 2.5 1.8 and 0.9 $\mu\mu c$ Sr^{90}/g Ca for the northern United States, the area between 60 10 N latitude and the world average respectively These values are still lower than those predicted from consideration of ecological discrimination factors which suggest that the over all decrease in Sr^{90} concentration in going from soils to human bone may be about 0.06 instead of 0.09 Assuming the data based on bone analyses are more applicable the average maximum bone equilibrium levels in about 1970 assuming no more bomb tests might approach 3.2, 2.6 and 1.7 $\mu\mu c$ Sr^{90}/g Ca for the northern United States the area between 60–10° N latitude and the world average respectively It is very important to point out that the above values are average maximum levels and because of individual variations a few people may approach five times these levels as an upper limit while a few will have only about one fifth the average

SIGNIFICANCE OF PRESENT AND PREDICTED STRONTIUM 90 LEVELS IN THE POPULATION

The maximum permissible level of Sr^{90} for persons employed in atomic energy work was set at 1 μc by the National and International Commissions on Radiological Protection This value was established by comparing the biological effects (on mice) of radiostrontium with those of radium The maximum permissible level for radium (0.1 μc) was established from the direct human experience of the radium dial industry The maximum permissible level of Sr^{90} for nonoccupational exposure or exposure of a large segment of the general population was set at 0.1 μc* by taking arbitrarily one tenth of the value for working personnel

The rationale behind a lower value for the general population is based on the numbers involved in the two groups at risk and the increased heterogeneity of the general population over that of the select working group The latter group is composed of supposedly healthy workers (over 20 years of age) while the general population group may contain children pregnant women the undernourished the sick and the old

The significance of the general hazard of present and predicted levels of Sr^{90} in bone can be evaluated only in relation to human experience which is indeed inadequate Bone sarcoma has resulted from a fixed skeletal burden of 3.6 μc of pure Ra^{226} and nondeleterious bone changes have been observed in persons having only 0.4 μc for a period of 25 years Necrosis and tumors of the bone have occurred also several years after large doses of X ray and human

* There is about 1 kg of calcium in the adult human skeleton therefore the MPL of Sr^{90} in the general population is equivalent to 0.1 μc Sr^{90}/kg Ca = 100 $m\mu c$/kg Ca = 100 $\mu\mu c$/g Ca By expressing the MPL in $\mu\mu c$/g skeletal Ca it is independent of the size of the skeleton

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Assuming an average of 20 g available Ca ft² of soil to a depth of 2½ in 1 mc of Sr^{90}/mile² is equivalent to 1.8 $\mu\mu c$ Sr^{90}/g available soil calcium Multiplication of the Sr^{90} surface deposition levels given earlier by 1 8 gives the Sr^{90} activity per gram of available soil calcium Multiplication of the specific activity of the available soil calcium by the Sr^{90} discrimination ratio of 0 09 should give the average maximum specific activity of calcium laid down in the adult skeleton through exchange and bone remodeling during the period of environmental contamination and the maximum Sr^{90} concentration in the bones of children who may be approaching an equilibrium state

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Attempts are being made (using radioactive tracers) to determine the over all Sr^{90}/Ca discrimination ratio in going from soils to human bone by indi vidually determining the discrimination factors (DF) that occur at the various steps along the ecological chain

The discrimination factor most difficult to establish is that from soils to plants (DF_1) It is dependent among other things on type of soil available soil calcium type of plant and perhaps on rainfall all of which may vary greatly with geographic location The soil to plant discrimination factor has been estimated as about 0 7 i e $\frac{(Sr^{90}/Ca)_{plant}}{(Sr^{90}/Ca)_{soil}} = 0\ 7$

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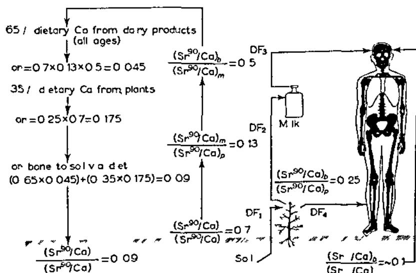


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The over all ratio ($OR_{bone-soil}$) in going from soil to human bone via the diet may be estimated from the various discrimination factors and the fraction of dietary calcium derived from dairy products and directly from other sources The Department of Agriculture on the basis of United States retail sales has

experience with X and gamma radiation suggests that about 80 roentgens may double the incidence of leukemia

The only other human experience with which present and predicted levels of Sr^{90} may be compared is that arising from natural background radiation Natural background dose to the bone (during a 70 year lifetime) may vary from about 8 to 38 roentgen equivalents of X or gamma radiation The major contribution to background variation is differences in the radium levels of soils and minerals

The maximum permissible level of Sr^{90} (100 $\mu\mu c/g$ Ca) is estimated to deliver about 10 to 20 roentgen equivalents to the skeleton during a 70 year lifetime This is comparable to the average natural background dose to the bone for the same time period and a factor of 2–3 below the maximum natural back ground dose to which small segments of the general population may be ex posed as a result of differences in altitude and natural radium content of soils and minerals It is a factor of 40 below the lowest skeletal dose which has produced minimal nondeleterious bone changes These data suggest that the present average maximum Sr^{90} equilibrium level in children will result in a lifetime radiation dose of approximately 2% of the accepted maximum per missible level for the general population The predicted average maximum level of Sr^{90} (from bone data) in about 1970 assuming no further weapons tests corresponds to a skeletal radiation dose of about 2 6% of the maximum permissible level with a spread of about 0 5 to 6%

The biological significance of present and predicted Sr^{90} average maximum equilibrium levels and maximum permissible levels for occupational and non occupational exposures is summarized in Table I

TABLE I

Biological Significance of Present and Predicted Sr^{90} *Average Maximum Equilibrium Levels and Maximum Permissible Levels for Occupational and Nonoccupational Exposure*

Sr level	MPL nonocc exp (100 $\mu\mu c/g$)	MPL occ exp (1000 $\mu\mu c/g$)	Min bone changes	Min sarcoma dose	Leukemia doubling dose
Present (1 8 $\mu\mu c/g$ Ca)	$\frac{1}{50}$	$\frac{1}{500}$	$\frac{1}{2000}$	$\frac{1}{20\,000}$	$\frac{1}{500}$
Predicted (2 6 $\mu\mu c/g$ Ca)	$\frac{1}{40}$	$\frac{1}{400}$	$\frac{1}{1400}$	$\frac{1}{14\,000}$	$\frac{1}{360}$
100 $\mu\mu c/g$ (MPL nonocc exp)	—	$\frac{1}{10}$	$\frac{1}{40}$	$\frac{1}{400}$	$\frac{1}{10}$
100 $\mu\mu c/g$ (MPL occup exp)	10	—	$\frac{1}{4}$	$\frac{1}{40}$	1

bone samples do not represent equilibrium conditions since much of the skeleton (especially of adults) was formed before fallout from bomb tests started When the bone analyses are converted to equilibrium conditions they suggest that equilibrium levels in the fall of 1956 should be about 2 5 1 8 and 0 9 $\mu\mu c$ Sr^{90}/g Ca for the northern United States the area between 60 – 10 N latitude and the world average respectively These values are still lower than those predicted from consideration of ecological discrimination factors which suggest that the over all decrease in Sr^{90} concentration in going from soils to human bone may be about 0 06 instead of 0 09 Assuming the data based on bone analyses are more applicable the average maximum bone equilibrium levels in about 1970 assuming no more bomb tests might approach 3 2, 2 6 and 1 7 $\mu\mu c$ Sr^{90}/g Ca for the northern United States the area between 60 –10° N latitude and the world average respectively It is very important to point out that the above values are average maximum levels and because of individual variations a few people may approach five times these levels as an upper limit while a few will have only about one fifth the average

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The rationale behind a lower value for the general population is based on the numbers involved in the two groups at risk and the increased heterogeneity of the general population over that of the select working group The latter group is composed of supposedly healthy workers (over 20 years of age) while the general population group may contain children pregnant women the undernourished the sick and the old

The significance of the general hazard of present and predicted levels of Sr^{90} in bone can be evaluated only in relation to human experience which is indeed inadequate Bone sarcoma has resulted from a fixed skeletal burden of 3 6 μc of pure Ra^{226} and nondeleterious bone changes have been observed in per sons having only 0 4 μc for a period of 25 years *Necrosis and tumors of the bone* have occurred also several years after large doses of X ray and human

* There is about 1 kg of calcium in the adult human skeleton therefore the MPL of Sr in the general population is equivalent to 0 1 μc Sr^{90}/kg Ca = 100 $m\mu c$/kg Ca = 100 $\mu\mu c$/g Ca By expressing the MPL in $\mu\mu c$/g skeletal Ca it is independent of the size of the skeleton

Theoretically the total yearly injection rate should be that amount which at equilibrium will not result in a significant fraction of the population ex ceeding the limit of safety If a constant yearly injection rate of 1 10 or 100 MT of fission is adhered to in about 100 years the amount of Sr^{90} added to the environment will come into equilibrium with the rate of Sr^{90} decay and con tinuation of weapons testing at that rate will result in no further increase in the average maximum equilibrium bone level of the population At that time the average equilibrium bone level will be directly proportional to the yearly injection rate i e if 10 or 100 megaton equivalents are injected per year the average equilibrium bone level will be 10 and 100 times higher respectively than it will be if only 1 megaton equivalent is injected

During the past 5 years weapons testing by *all nations* has averaged about 10 megaton equivalents of fission per year Elaborate and detailed mathe matical calculations predict that average soil and bone levels will be about 11 times higher than at present when equilibrium with the current rate of testing is reached in about 100 years Thirty years of testing at the present rate should result in average soil and bone levels about five times the present levels Continued testing of fission weapons at the present rate for 100 years might result in average maximum equilibrium bone levels of about 30 25 and 12 $\mu\mu c$ Sr^{90}/g Ca for the United States the area between 60 –10 N latitude and the world respectively The upper limit that might be expected in the United States assuming a factor of 5 is required to make allowances for nonhomo geneities of Sr^{90} deposition and uptake would approach about 150 $\mu\mu c$ Sr^{90}/g Ca or 150% of the accepted maximum permissible level After 30 years of testing average maximum equilibrium bone levels may approach one half of the above values which may result in an upper limit for the United States of about 75% of the maximum allowable level Assuming the average yearly injection of fission products during the past 5 years was equal to approxi mately 10 MT of fission yield then testing of 10 MT of fission per year for 30 years should probably be considered the upper limit or 5 MT of fission yield per year assuming testing for infinite time If these are the limits of acceptable injection rate international agreement not to exceed these levels seems desir able Present levels of Sr^{90} contamination are due almost entirely to tests held by only two nations Present and predicted future Sr^{90} levels even if weapons tests are continued at the present rate for a few years does not seem dangerous However indiscriminate testing of high fission yield weapons by many nations could result in serious levels of world wide contamination

SUMMARY

Evaluation of existing data supports the following general conclusions

(1) Radioactive isotopes deposited in the bone in sufficient quantity will produce serious consequences including bone cancer and leukemia Present

An interesting comparison in Table I is that between Sr^{90} levels and the leukemia doubling dose assuming a nonthreshold relation between leukemia incidence and radiation exposure These data indicate that the predicted average maximum equilibrium level of Sr^{90} assuming no more weapons tests after the fall of 1956 is 1/360 of the leukemia doubling dose Theoretically this level is equivalent to an increase in leukemia incidence of 1 7 cases per 10 million population The rate in some localized areas may be 3 or 4 times higher but averaged over the United States population of 165 million this would produce an increased leukemia burden of about 30 cases per year If the entire world population is allowed to reach an average maximum Sr^{90} equilibrium level of 100 $\mu\mu c/g$ Ca the average increase in the world s leukemia burden would be about 16 000 cases per year or an increase of about 10%

The above estimates actually may be pessimistic since they entail the as sumption that Sr^{90} beta radiation induces leukemia of bone marrow origin at the same rate (per unit of absorbed dose) as X and gamma rays Much of the beta radiation from Sr^{90} will be absorbed in the bone and not reach the hema poietic tissues at all Experiments on mice suggest that Sr^{90} may not produce leukemia as effectively as X rays Furthermore leukemia was not a significant finding in the radium dial painters

Human data on radiation induced osteogenic sarcoma are not adequate to provide a basis for a sarcoma doubling dose or for an estimation of the per cent of normal incidence attributable to natural background If however the same assumptions used for leukemia are applied to osteogenic sarcoma (non threshold response 10% of normal incidence of 2 per 100 000 attributable to natural background and a doubling dose of 80 rads) the predicted average maximum Sr^{90} equilibrium level from weapons already tested would produce an increase in the United States burden of osteogenic sarcoma of about 10 cases per year If the world population is allowed to reach an average maxi mum Sr^{90} equilibrium level of 100 $\mu\mu c/g$ Ca the bone tumor incidence would be increased by about 5000 cases

It should be re-emphasized that the above considerations are extremely tenuous and are based on the questionable assumption that the incidence of leukemia and bone sarcoma bear a linear relationship to radiation dose This is taking the most pessimistic view by saying any amount of radiation is bad *and that there is no absolutely safe dose*

STRONTIUM 90 LEVELS IN RELATION TO FUTURE WEAPONS TESTS

The most important question regarding the potential hazard of world wide fallout to the general population is its relation to future weapons testing If there is an upper limit to the amount of Sr^{90} that can be tolerated in the bones of the population then the number of megaton equivalents of fission products that can be contributed per year to the biosphere by all nations must be limited

mended levels For this reason international agreement to limit testing might be desirable while negotiating for international agreement to stop testing altogether

(4) The data presently available definitely show that the greatest question concerning world wide Sr^{90} contamination concerns the decision as to the *acceptable* maximum permissible body dose for the general population in terms of the individual and in terms of world health The answer to this question involves moral and humanitarian principles as well as scientific uncertainties as to the biological consequences

Some deductions as to the worst biological consequences of Sr^{90} fallout (which excludes the genetics question) can be made by accepting two rather pessimistic assumptions neither of which has been proved but both of which seem conservative and reasonable The first assumption is that there is no *absolutely safe* radiation dose and any amount is theoretically bad The second assumption is that about 10% of the normal incidences of bone tumors and leukemia are due to natural background radiation

On the basis of these assumptions weapons tests to date might conceivably increase the incidence of leukemia in the United States from 10 000 cases per year to about 10 030 and the incidence of bone cancer might increase from about 3 000 to about 3 010 This suggests a total increase of forty cases out of a population of 165 million people as the maximum biological consequence of Sr^{90} fallout from weapons tested to date *by all nations* If the present rate of biospheric Sr^{90} contamination continues for 30 years the biological consequence of Sr^{90} fallout may be a total increase in the United States population of 250 cases per year of these two diseases There is a good chance that this prediction may overestimate the population risk The assumption that even the smallest amount of Sr^{90} deposited in bone carries a small probability of harm has not been definitely proved yet and there is still a possibility that a threshold exists below which no leukemia and bone cancer will be produced

(5) The present data also indicate that much more research on both the physical and biological factors of fallout must be done if weapons tests are to be continued This research is necessary to narrow the limits of error and uncertainty in existing data and to permit predictions to be based on facts instead of on what appear to be reasonable assumptions More research on the biological and medical effects of radiation and radioactive materials is essential even to the future of the power reactor program which also produces Sr^{90} and other fission products

Although present knowledge of the biological effects of radiation and radioactive materials is not all it should be to allow plunging ahead recklessly and without worry into all aspects of nuclear technology it is adequate to dispel an attitude of gloom and doom Radiation is not the only potential hazard

Sr^{90} levels in the bones of the population are quite low The present average maximum Sr^{90} radiation dose to the bones of young children is greater than that for adults and is equal to about 2% of the average dose received from the unavoidable natural background radiation contributed by cosmic rays and by radium thorium uranium etc, in the environment The present level is about 2% of the maximum permissible value adopted by the National and International Commissions on Radiological Protection as acceptable for large segments of the general population The present Sr^{90} radiation dose to adults averaged over the total skeleton is only about one tenth of that for children Because of nonuniformity of fallout and individual variation in uptake and deposition of Sr^{90} in bone a very small number of people may accumulate a skeletal dose that will be about five times the average and an equal number will accumulate only about one fifth the average Since in the stratosphere there is still some Sr^{90} from past weapons tests the average radiation dose may continue to rise until about 1970 even if no more weapons tests are held At that time the equilibrium level may be about 3–4% of the average natural background

(2) If Sr^{90} contamination from weapons testing by all nations continues at the same rate as has occurred during the past 5 years (about 10 megaton equivalents of TNT fission yield per year) equilibrium will be reached in about 100 years At equilibrium the amount of Sr^{90} which will disappear each year from our environment due to radioactive decay will just about equal the amount that is being produced each year At this time continuing weapons tests will not result in any further increase in Sr^{90} in the bones of the population At this theoretical limit (which would be reached in about 2050) the average Sr^{90} radiation dose to the bones of the population of the United States may be about 30% of the average radiation dose from natural background or about 30% of the maximum permissible level adopted by the National and International Commissions Since individual variations may result in a small number of people accumulating Sr^{90} burdens that are 5 times the average the radiation dose to these people may approach 150% of the recommended maximum level as an upper limit Thirty years of testing may result in average levels one half the equilibrium values This may result in a few people in the United States approaching body burdens about 75% of the recommended maximum On this basis limitation of fission weapons testing to 10 megatons per year for 30 years or about 5 megatons per year indefinitely might be desirable

(3) Existing data support the conclusion that the present rate of biospheric Sr^{90} contamination if continued for several years will not produce population bone levels that will exceed the maximum permissible levels accepted by the National and International Commissions on Radiological Protection The data also show that many nations cannot test weapons indiscriminately and indefinitely without running the risk of seriously exceeding these recom

1 DISEASES OF REPRODUCTION IN MALE AND FEMALE

man is facing as part of the price of living in a highly developed society, nor is it the insurmountable one

What does the accompanying mass of technical data mean with regard to the controversy over cessation or continuation of nuclear weapons tests? Nowhere in this report has a recommendation been made either to stop or to continue testing Such a recommendation requires a careful weighing of the importance of the nations nuclear weapons capability in averting a nuclear war against the probability that a few people who otherwise might not have done so might get leukemia or bone sarcoma or manifest a genetic abnormality Decision to stop or continue tests therefore requires a value judgment involving knowledge of the potential seriousness of present and future threats to the national security whether they should or should not be stopped on the basis of moral and humanitarian principles is not readily amenable to solution by the scientific method.

NEW ASPECTS OF VIBRIOSIS IN SHEEP

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Vibriosis an acute infectious disease in sheep is characterized by abortion during advanced stages of gestation and is caused by *Vibrio fetus* Infected flocks maintained in sheds or pasture prior to lambing may incur an incidence of abortion as high as 70% and of mortality of aborting ewes as high as 5%

During the past 4 years ovine vibriosis has been studied intensively in a western regional research project by investigators of California Colorado Idaho Montana Utah Washington and Wyoming Important information pertinent to etiology transmission and immunity has been obtained and will be presented at this time

ETIOLOGY

The genitalia of sheep contain two species of *Vibrio* *V fetus* is consistently present in aborted fetuses membranes and uterine exudates from aborting ewes *V fetus* has been found rarely if ever as a natural infection in the genitalia of rams or in the genitalia of ewes after cessation of exudation following abortion

Another species of *Vibrio* officially unnamed but for which the name *Vibrio dubius* was recommended by a special subcommittee of Western Regional Research Project W 27 has been isolated from the prepuce of rams by Binns (1954) Miller (unpublished) and Firehammer (1955) and from the vagina of ewes by Firehammer This organism corresponds closely to the non pathogenic vibrio of cattle genitalia as described by Bryner and Frank (1955) by not producing catalase and by generating hydrogen sulfide *in vitro* Although its pathogenicity for sheep has not been studied exhaustively available evidence indicates that this organism is nonpathogenic

Miller (1957) studied 13 pregnant ewes which had been fed *Vibrio fetus* infected tissues Blood cultures prepared at 2 day intervals prior to and following oral inoculation disclosed a bacteremia which persisted from 2 to 8 days following inoculation in 6 ewes

TABLE III

Transmission of Vibriosis to Ewes in the Fifth Month of Gestation 1957

	Ewes		
Lots	Number	Abortions	Oral inoculation
1	25	12 (48%)	infected tissue
2	22	2 (9%)	none

In oral transmission experiments conducted 1955 and 1956 33 ewes aborted The mean interval in days between inoculation and abortion was 13 2±4 42

Experimental attempts to transmit vibriosis by coition have been unsuccessful In the experiment presented in Table I above rams which originated from an infected flock and which had been inoculated intrapreputially with viable *V fetus* immediately prior to breeding failed to transmit the disease Firehammer Marsh and Tunnicliff (1956) inseminated 16 ewes with a mixture of normal ram semen and a culture of viable *V fetus* No abortions occurred

These experiments demonstrated conclusively the transmissibility of vibriosis to ewes in the fifth month of gestation by oral inoculation and direct attention to the importance of strict sanitation to avoid contamination of feed and water with infected discharges from aborting ewes

IMMUNITY

The concensus from the study of natural outbreaks is that ovine vibriosis tends not to recur in the same flocks during years immediately subsequent to the disease The failure of recurrence under natural conditions may result from immunity established by the disease or from lack of exposure An experiment conducted in 1956 was designed to test immunity from incidence of the disease and from oral exposure of nonpregnant ewes to *V fetus* infected lamb tissues One hundred and nine ewes were utilized in three lots Lot 1 contained 21 normal ewes Lot 2 contained 44 ewes each of which aborted from vibriosis on the previous year Lot 3 contained 44 ewes each of which was inoculated orally with *V fetus* infected tissue during the previous summer when the ewes were nonpregnant yearlings During the fifth month of gestation each ewe of the three lots was challenged by oral inoculation with *V fetus* infected tissues Seventy six per cent of the ewes of the control lot aborted while 9% of the ewes which developed vibriosis on the previous year aborted and 7% of the ewes which were fed infected tissues as nonpregnant yearlings aborted The difference for number of abortions is highly significant ($P<0\ 01$) These data are presented in Table IV

The induction of immunity by feeding *V fetus* infected tissues to non pregnant yearlings suggested the possibility of immunization by artificial

TRANSMISSION

The transmissibility of vibriosis by oral inoculation and by coition has been investigated In an experiment conducted in 1955 152 ewes were arranged into three lots Lot 1 received no treatment lot 2 was bred by rams which originated from infected flocks and which had been injected intrapreputially with pathogenic viable *V fetus* while lot 3 was bred by clean rams and was inoculated orally during the fifth month of gestation with *V fetus* infected tissues from an aborted lamb No abortions occurred in lots 1 and 2 while 73% of the ewes of lot 3 aborted These data are shown in Table I Statistically the difference for the number of abortions for the lots is highly significant ($P<0\ 01$)

TABLE I

Transmission of Vibriosis to Ewes 1955

Lots	Ewes			Rams
	Total	Dead lambs	Inoculation	
1	61	0	none	clean
2	68	0	none	infected
3	23	17 (73%)	infected tissue	clean

In another experiment conducted in 1956 76% of the ewes of a lot inoculated orally with infected tissues aborted while 8% of a similar lot of uninoculated ewes aborted The difference for the number of abortions for the lots is highly significant ($P<0\ 01$) These data are presented in Table II

TABLE II

Transmission of Vibriosis to Ewes in Fifth Month of Gestation 1956

Lots	Ewes		
	Number	Dead lambs	Oral inoculation
1	21	16 (76%)	infected tissue
2	25	2 (8%)	none

In an experiment conducted 1957 48% of the ewes of a lot inoculated orally at the beginning of the fifth month of gestation with infected lamb tissues aborted while 9% of the ewes of a similar lot uninoculated aborted These data are presented in Table III and contain statistically significant difference ($P<0\ 05$) for number of abortions

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TABLE IV

Immunity to Vibriosis 1956

Lots	Ewes			Oral challenge inoculation
	Status	Number	Abortions	
1	Normal	21	16 (76°)	Infected tissue
2	Aborted 1955	44	4 (9 1%)	Infected tissue
3	Fed infected tissue 1955	44	3 (6 8%)	Infected tissue

means Four vaccines viable *V fetus* propagated on chicken egg viable cultures of *V fetus* recently isolated formalinized culture of *V fetus* and formalinized infected tissues were prepared Each vaccine was administered parenterally to the ewes of an experimental flock prior to the breeding season During the fifth month of gestation each animal was challenged by oral inoculation with *V fetus* infected lamb tissues Abortion rates for vaccinated lots did not differ significantly ($P>0\ 05$) from the non vaccinated lots These data are presented in Table V

TABLE V

Vaccinated Ewes Challenged by Oral Inoculation with Infected Tissue during Fifth Month of Gestation

Lots	Vaccines	Ewes	% abortions
1	None	25	48
4	Egg propagated *V fetus*	24	62
6	Formalinized infected tissue	24	33
7	Formalinized culture *V fetus*	21	62
8	Viable culture *V fetus*	19	65

SUMMARY

The prepuce of some rams contained a nonpathogenic vibrio species which did not produce catalase and did generate hydrogen sulfide while many aborted lambs contained pathogenic *V fetus* which produced catalase and failed to generate hydrogen sulfide

Experimentally vibriosis was transmitted to ewes in the fifth month of gestation by feeding infected tissue from aborted lambs Vibriosis was not transmitted by rams at coition

Occurrence of vibriosis and feeding of *V fetus* infected lamb tissues induced immunity in ewes for 1 year at least

in the organism might occur during its growth in animals and passage from one animal to another

Kuzdas and Morse (1956) questioned the advisability of identifying *Vibrios* isolated from the reproductive tracts of sheep and cattle as being pathogenic on the basis of catalase production alone *Vibrios* which had been isolated from soil water and cheese were found to be catalase positive

Bond (1957) isolated catalase positive smooth colonies from a culture of bull semen which had been classed as catalase negative It is possible that both types were present in the original culture and that it was not a change of catalase activity of a pure culture of *Vibrio*

Bryner and Frank (1955) reported that the catalase positive pathogenic and catalase negative nonpathogenic types could usually be identified by the color and physical characteristics of the growth on blood agar The pathogenic type occurred as white colonies or spreading growth with a peripheral bluish hue The nonpathogenic type generally occurred as a brownish green spreading growth with a metallic sheen and occasionally produced partial hemolysis

Ristic Herzberg and Sanders (1956) studied colonial and cellular variations of ten strains of *Vibrio fetus* streaked on solid Albimi media containing 2% agar By using oblique indirect transmitted light they were able to detect five types of colonies including smooth rough cut glass mucoid and very rough The non smooth variants apparently grew as well under normal aerobic conditions as in an atmosphere of 10% CO_2 Organisms from the smooth colonies failed to grow under normal aerobic conditions They suggested that this factor would favor the propagation of the non smooth variants under laboratory conditions The smooth variants were more strain specific in their agglutinability than the non smooth variants The mucoid variants were the least specific In regard to agglutinability of variants they pointed out that cell morphology under the conditions of their experiment was not a sufficient criterion for determining the predominant variant type The long filament forms appeared to be serologically closer to the smooth forms than the relatively short forms of the very rough type

Bond (1957) reported three main types of colonies including smooth rough and mucoid The mucoid form was significantly different biochemically and the variants were catalase negative produced hydrogen sulfide and grew in deep stab cultures On the other hand the smooth and rough forms were catalase positive did not form hydrogen sulfide and did not grow in deep stab cultures All variants reduced nitrates to nitrites

DIAGNOSIS

Diagnostic procedures have been improved during the past several years so that it is not difficult to establish a diagnosis of vibriosis on a herd basis This may be accomplished most easily by testing vaginal mucus from approximately

NEW ASPECTS OF VIBRIOSIS IN CATTLE

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THE literature on vibriosis of cattle was reviewed comprehensively by Plastridge in 1955 Consequently this paper will deal primarily with material which has been published since his review

Bovine vibriosis is a widespread venereal disease of considerable economic importance The clinical manifestations are well known and will not be discussed The areas which require more study are the characteristics of *Vibrios* recovered from bovine genital organs and the diagnosis pathogenicity transmission control and treatment of the disease

CHARACTERISTICS OF BOVINE GENITAL VIBRIOS

Bryner and Frank (1955) suggested the use of the catalase test for differentiating *Vibrio fetus* from non pathogenic vibrios isolated from the reproductive tract of cattle The only type of *Vibrio* which they isolated from aborted fetuses was catalase positive did not produce hydrogen sulfide and grew on or near the surface of deep stab cultures in thiol medium plus 0 5% agar The other type of *Vibrio* was not associated with infertility It was catalase negative produced hydrogen sulfide and grew deep in stab cultures Both types reduced nitrates The catalase test appears to be a very useful yet simple test for screening vibrios for pathogenicity Frank Bryner and Caruthers (1956) isolated catalase negative vibrios from virgin bulls but not from virgin heifers They reported that the catalase negative *Vibrio* was spread infrequently by natural service and that the infection was of short duration in females and persistent in bulls This organism did not appear to affect fertility

Akkerman Terpstra and van Waveren (1956) reported a third type of *Vibrio* which was catalase positive and formed hydrogen sulfide It was isolated from aborted fetuses but not from bulls or infertile cows Fifteen out of 20 strains isolated from aborted fetuses were classified in this group

Although no one has demonstrated that catalase negative vibrios are pathogenic for cattle the possibility of them becoming virulent under certain circumstances should not be ignored It is known that *Vibrio fetus* undergoes rather rapid changes when kept in the laboratory It is possible that alterations

in the organism might occur during its growth in animals and passage from one animal to another

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DIAGNOSIS

Diagnostic procedures have been improved during the past several years so that it is not difficult to establish a diagnosis of vibriosis on a herd basis This may be accomplished most easily by testing vaginal mucus from approximately

six–eight heifers suspected of harboring the infection The mucus gives a positive agglutination reaction in approximately 40–80 days following exposure and the titer persists for approximately 7 months Thus it is suggested that mucus samples be taken from heifers which have been bred to suspected carrier bulls for the first time at least two months but not over nine months previously It is preferable to use mucus from heifers because an occasional cow may carry a mucus titer for several years

Detection of the presence of *Vibrio fetus* in individual animals still presents a problem especially in regard to bulls

Although techniques for isolating *Vibrios* directly from the prepuce and semen of bulls have been greatly improved virgin heifers must still be used as test animals for detecting the presence or absence of the organism in bulls Adler (1957) relied on a single test heifer He collected two or three ejaculates in immediate succession in an artificial vagina The prepuce was then washed out with 200 ml of 0 9% sodium chloride solution adjusted to pH 7 5 The semen and preputial washings were centrifuged separately at 3500 r p m for 20 minutes The centrifugates were mixed and 1–5 ml infused with a glass pipette into the cervix of a heifer during estrus He considered semen to be the most essential portion of the test material and the preputial washing fluid as a supplement The first sample for culture was collected from the heifer 2–5 days following inoculation Up to six samples were collected at 3–4 day intervals Material was obtained from the external and internal cervical os by means of two modified Folmer Nielsen biopsy instruments inserted via the vagina in a brass tube He found that sampling the two areas resulted in a higher recovery rate He was often able to recover pure growths of *Vibrio* from the biopsy material but seldom from mucus collected from the vagina In a series of fifteen bulls which were tested several times one was positive on the first test negative on the next, and positive on the third Two were negative on the first trial and positive on the second Another bull was positive on the first test and negative on three subsequent tests *Vibrio* was recovered from five bulls on each attempt *Vibrio* was not isolated from six of the fifteen bulls It would appear that these results should create considerable doubt regarding reliance upon a single test heifer for detection of the *Vibrio* status of a bull According to Terpstra (1956) the mating of a virgin heifer did not give an absolute guarantee of the *Vibrio* status of a bull when the test mating failed to reveal *Vibrio* Lawson (1956) also questioned the use of a single heifer for testing the *Vibrio* status of a bull

Hughes (1956) was able to recover *Vibrios* from 256 (37 9%) of 675 ejaculates in one stud and 54 (25%) of 216 ejaculates from another stud All of the ejaculates were from bulls from which *Vibrios* had been recovered at least once In an effort to determine the number of cultural attempts necessary to make a positive diagnosis he cultured semen from 64 bulls from which *Vibrio* had been isolated at least once previously Twenty five isolations were made

on the first culture fifteen on the second From three to fourteen cultures were required to isolate *Vibrio* from the rest of the group

Adler (1957) was very successful in recovering *Vibrios* from the female reproductive tract Thirty two heifers were infected with *Vibrio* cultures By use of modified Folmer Nielsen instruments material was collected from the external and internal cervical os of the inoculated heifers Cultures of 184 samples yielded 73% *Vibrio* recovery from external os samples and 77% from the internal cervical os A comparison was made of the recovery rate from vaginal and cervical secretions from eleven heifers Eighty-eight per cent of the cervical specimens yielded *Vibrios* while recovery was possible in only 34% of the vaginal specimens It was also pointed out that contamination of vaginal material was much higher (65%) than cervical material (4%)

Unfortunately laboratory animals have not been shown to be very useful in the diagnosis of vibriosis The high cost of using heifers as test animals should stimulate more research on the use of small animals for diagnostic purposes Robinson van Rensburg van Heerden and van Drimmlen (1956) reported the isolation of *Vibrio* from guinea pigs which were injected intraperitoneally with bull semen These workers injected 0 1 ml semen and euthanized the guinea pigs 5–7 days later Since the guinea pig method was not used on a large number of samples they were not able to evaluate the procedure

Most authorities agree that vaginal mucus is more useful than blood serum for detecting *Vibrio* agglutinins in females One of the disadvantages of the test listed by Plastridge (1955) was the failure of some infected animals to react to the test These failures may be partially due to the time of sampling in relation to the exposure time and the stage of the estrous cycle It can also be due to the choice of antigens Vandeplassche *et al* (1956) stated that *Trichomonas fetus* infection seriously interferes with the mucus agglutination test for vibriosis They were frequently dealing with dual infections

There is urgent need for a standardized antigen which is tested periodically to make certain that it does not change in sensitivity If this could be developed and made universally available there would probably be fewer discrepancies in the reports from various laboratories

PATHOGENICITY

Surprisingly few reports have been published on the pathological effects of *Vibrio fetus* on the tissues of the reproductive tract of cattle Simon and McNutt (1957) reported salpingitis and cervicitis in four heifers which were slaughtered following intrauterine inoculation of a mixture of four or five different *Vibrio* strains They were not able to culture *Vibrio* organisms from these heifers The relative absence of endometritis in these heifers is difficult to understand We have observed endometritis in the majority of our recently infected heifers They also studied the pathological effects in pregnant heifers of intrauterine and intravenous injections of *Vibrio* Endometritis salpingitis

and extensive inflammatory edema of the fetal membranes were reported as the prominent lesions resulting from these inoculations

The persistence of the organism in the female reproductive tract presents a problem in regard to control of the disease Frank (1956) reported that one heifer remained infected for 464 days Adler (1957) found that one of his experimental heifers retained the infection for 401 days It would be worth while to know the relationship between the persistence of the organism and persistence of antibody titer Frank (1956) obtained samples periodically for culture from 42 infected females in five herds He demonstrated *Vibrio* in 76% of the females for only 40 days in 12% for 2–6 months and in the rest for 7–12 months

Vibrios were recovered from slaughtered animals by Mundt (1955) from the vagina cervix uterus and oviducts but only from the prepuce penis and anterior portion of the urethra of bulls

Little is known about the persistence of immunity Vandeplas che *et al* (1956) stated that reinfection of females may occur within 2–3 months after recovery from the first infection Frank (1956) reported that immunity is indefinite

Vibrio infection of the female reproductive tract of cattle causes failure of conception embryonic death and abortion at any stage of pregnancy Non return rate and embryonic mortality in cows artificially inseminated with semen from *Vibrio fetus* carrier bulls were studied in an experiment by Willet *et al* (1955) They found that embryonic mortality was significantly greater and the non return rate lower among cows inseminated with semen from *Vibrio fetus* carrier bulls when no antibacterial agents were added to the ex tender as compared to when they were present The details of the experiment will be mentioned under control of the disease

TRANSMISSION

It is generally agreed that genital vibriosis of cattle is a venereal disease There is some disagreement regarding the possible methods of transmission No one disputes that it is usually spread by natural service to *Vibrio* carrier bulls or by artificial insemination with non antibiotic treated semen from carrier bulls Plastridge *et al* (1955) maintained that the disease spread by contact in their experimental heifers Since other workers do not have experi mental evidence indicating that contact spread can occur more work probably should be conducted on this phase of the *Vibrio* problem

Hughes (1956) and Adler (1957) have presented evidence indicating that vibriosis spreads from one bull to another within artificial breeding studs The incidence of vibriosis in bulls which had been used for natural service and those which had been used only artificially was determined in one stud by Hughes (1956) He reported an incidence of 56 4% of vibriosis in 39 bulls which had never been used naturally as compared to 56 7% in bulls which had served

naturally Frank (1956) observed the apparent spread of vibriosis following the transfer of a clean bull into a stall previously occupied by an infected bull Detailed studies are necessary to clarify the possible methods of dissemination of the disease within bull studs

CONTROL

At present the easiest method in our experience for controlling vibriosis is by artificially inseminating with semen from non infected bulls or semen to which 500 units of penicillin and 500 units of streptomycin have been added to each milliliter of diluted semen The semen should be extended at least 1 25 and held for at least 6 hours at refrigerator temperature before use Adler (1957) inseminated 45 heifers with semen from *Vibrio* carrier bulls The semen was diluted ten times and approximately 600 units of streptomycin was added to each milliliter of diluted semen Vibriosis was not diagnosed in any of these heifers Twenty two out of 24 controls developed the disease These experiments suggest that streptomycin alone is effective and that a dilution of only ten times is adequate Since semen can be diluted much more than twenty five times without effecting fertility and dilution of semen alone reduces the incidence of infection we do not believe that a lower extension rate should be used

Willet *et al* (1955) have clearly demonstrated the effect on fertility to streptomycin treatment of semen from *Vibrio fetus* carrier bulls Semen from seven catalase positive *Vibrio* carrier bulls was used to inseminate 9810 cows Four semen treatments were compared including (1) no antibiotics (2) 0 3% sulfanilamide plus 500 units of dihydrostreptomycin sulfate per milliliter (3) 500 units of dihydrostreptomycin sulfate per milliliter and (4) 500 units of potassium penicillin G plus 500 units of dihydrostreptomycin sulfate per milliliter Approximately equal numbers of cows were inseminated with semen from each treatment group No significant differences in non return rates were detected amongst the three antibiotic treatments The cows inseminated with non antibiotic treated semen had approximately an 8% lower 28–35 day non return rate and approximately a 17% lower 60–90 day non return rate than cows inseminated with antibiotic treated semen It is apparent that the use of semen from *Vibrio fetus* carrier bulls resulted in markedly greater differences in non return rates when no antibacterial agents were added than when they were included in the extender Only one of the bulls with unknown or non pathogenic *Vibrios* had such a decline in non return rate Consequently it appears that the most beneficial effect of treating semen with streptomycin is the control of *Vibrio fetus* It is quite probable that vibriosis was widely disseminated by means of artificial insemination before antibiotics were added to semen

As far as we know a suitable vaccine which will control bovine vibriosis has not been developed There probably should be some reservation as to the use

of a vaccine if an effective product is eventually developed Since vibriosis of cattle is a venereal disease it might be better to attempt to eliminate the disease instead of trying to live with it

TREATMENT

Adler (1957) has conducted rather extensive experiments on the treatment of bovine vibriosis He found lugols and Lotagen (dioxydimethyl diphenylmethanedisulphonic acid) to be ineffective as intrauterine therapy In his experiment the use of parenteral streptomycin in therapeutically active blood concentrations also failed to cure the disease in cows He concluded that intrauterine streptomycin therapy was the most effective treatment He stated that the optimum treatment was 1 g of streptomycin infused into the uterus daily for 3 days or 1 g repeated 48 hours later An aqueous vehicle was as effective as an oil suspension Since misplaced oil can produce granulomas in the cervix and uterus it seems that an aqueous vehicle would be preferable One group of his experimentally treated heifers will be mentioned to illustrate the effectiveness of his recommended treatment Thirty eight infected heifers were injected intrauterinely two times at 48 hour intervals with 1 g of strepto mycin in an aqueous vehicle Only one heifer remained infected following this therapy In his experience aureomycin was not as effective as streptomycin Streptomycin was as effective as a combination of streptomycin and penicillin The stage of the estrous cycle during which streptomycin was infused into the uterus apparently did not influence recovery

A completely satisfactory treatment for eliminating *Vibrio fetus* from bulls has not been perfected It is not known whether the reported failures are due to reinfection or therapy failure Before treatment of bulls can be properly evaluated rigid controls will have to be instituted to insure that the treated animals do not have an opportunity to become re exposed Adler (1957) has tried streptomycin subcutaneously and locally as well as streptomycin and penicillin locally He used 74 treatments on 62 bulls Subsequently the *Vibrio* organism was detected in 33 animals He did not know whether the treatments failed or whether the bulls became reinfected Hughes (1956) has had similar experience treating bulls in a stud

In treating cows and bulls for vibriosis most workers have chosen to ignore the possibility of the *Vibrio* organism in the treated animals becoming strepto-mycin resistant Since streptomycin is so widely used for treating semen and there is at least a theoretical possibility of *Vibrio fetus* becoming resistant to this antibiotic more thought should be given to the possibility of using other antibiotics for the treatment of animals

SUMMARY

1 The catalase test appears to be useful for screening *Vibrios* for patho-genicity

2 Catalase negative *Vibrios* have not been incriminated as a cause of infertility

3 There are at least three main types of colonial variants of *Vibrio fetus* They are smooth rough and mucoid

4 The diagnosis of vibriosis on a herd basis is not difficult Detection of the presence of *Vibrio fetus* in individual animals especially bulls presents a more difficult problem

5 Vibriosis causes cervicitis endometritis and salpingitis in cows but no demonstrable lesions in bulls

6 Little is known about the persistence of immunity

7 *Vibrio* infection of the female reproductive tract results in failure of conception embryonic death and abortion at any stage of pregnancy

8 Vibriosis may be spread either by natural service or artificial insemination with non antibiotic treated semen There is disagreement regarding the possibility of contact spread

9 The infection apparently spreads within bull studs

10 The disease may be controlled by artificially inseminating with streptomycin treated semen from *Vibrio fetus* carrier bulls

11 Cows may be treated fairly successfully with intrauterine infusions of streptomycin

12 The treatment of bulls has not been perfected

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CATARRHAL VAGINITIS OF CATTLE

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THE most severe economic losses suffered by the livestock industry are due either directly or indirectly to problems associated with reproduction The conditions involved range from mild genital infections accompanied by irregular estrous cycles and lowered conception rates to complete sterility in both the male and female animal Early fetal deaths and abortions also form part of the picture

While common to all types of cattle reproductive problems are of major importance insofar as dairy animals are concerned When it is realized that the annual losses in the United States due to reproductive disorders in dairy cattle alone amount to some quarter of a billion dollars the importance of these problems to the industry becomes highly realistic

It has long been recognized that many of the reproductive dysfunctions of cattle are of physiological origin resulting directly from deranged metabolism hormonal imbalance and even dietary factors In addition infections such as brucellosis vibriosis granular and vesicular vaginitis and trichomoniasis contribute significantly to the total loss

A syndrome characterized most consistently by a vaginitis and which appears to be associated with infertility has recently attracted considerable attention Whether this condition existed previously as one of the manifesta tions of infertility and has only lately gained prominence because a virus or viruses has been found associated with it or whether it is a new disease has not been determined However the fact that it was reported from four con tinents almost simultaneously would suggest that it is not only a widespread but a relatively new development

THE VAGINITIS SYNDROME IN CATTLE

In South Africa a disease of cattle resembling infectious epididymitis and vaginitis—known colloquially as epivag —was first reported from Kenya (Daubney *et al* 1938) and was encountered in 1949 in the northwestern part of the Transvaal province (McIntosh *et al* 1954) Over the next several years the disease spread into the Orange Free State and Natal and caused such severe economic losses among breeding cattle that artificial insemination had to be resorted to as a means of coping with it Fortunately this measure

was highly successful so that the disease now assumes much less importance

Field studies of the condition in South Africa revealed that bulls in herds containing affected cows did not always show evidence of disease This led some investigators to conclude that two conditions were involved epivag a venereally transmitted infection that produces clinical infection and sterility in both male and female cattle and a relatively mild vaginitis not necessarily related to breeding operations that occurs in both heifers and adult cows It is not clear whether this latter infection was considered to affect bulls at least as a clinically recognizable entity

A virus was subsequently isolated in embryonating hen eggs from the vaginal discharges of cattle in a herd in which no infection could be detected clinically in the bulls (McIntosh *et al* 1954) The isolate was shown to be capable of causing a vaginitis similar to although milder than that occurring under natural conditions While attempts were not made to infect bulls with the agent it was believed because of the absence of infected bulls in the herd involved that the isolation had been made from an outbreak of vaginitis rather than of epivag

While studying herd infertility problems in England Millar (1955) encountered in dairy cattle two apparently infectious forms of infertility unassociated with the recognized genital infections The main feature of the first and less severe of the two conditions was an acute vaginitis accompanied by a mucopurulent vaginal discharge The conception rate in herds containing cows thus affected fell sharply It appeared that this disorder was not necessarily spread by the venereal route since heifers on the same farm showed evidence of the infection a few days after it appeared in the mature females Millar does not state whether bulls are involved

The second syndrome observed by Millar differed from the first in that it produced a marked clinical infection in both males and females and it was venereally transmitted The usual outbreak history was that it occurred following the introduction of one or more animals of either sex from another herd The disease was characterized by postcoital and postpartum discharges a low conception rate irregular estrous cycles and evidence of early fetal deaths and abortions An acute vestibular vaginitis was a common although not a constant feature However an endometritis usually occurred and if not treated persisted in some cases for months The period of lowered fertility was relatively short lasting for 5 months or less The bulls in these herds apparently contracted the infection from affected cows at the time of service and subsequently developed inflammatory changes in the testicles which resulted in impairment in semen quality The semen was evidently infectious since uninfected cows bred to such bulls in turn developed the infection Infected bulls usually responded to treatment returning to their preinfection fertility level within 6 months

Millar succeeded in isolating a virus in chicken embryos from both the genital discharges of infected cows and the semen of infertile bulls The isolate on inoculation into heifers by the vaginal route reproduced the typical vaginitis and endometritis

Recently a virus has been isolated by McClure (1956) from cases of bovine vaginitis in herds in New Zealand in which the infertility rate was high McClure describes the condition as follows (McClure 1956)

> The common syndrome appears to be characterized by venereal trans mission purulent cervico-vaginal mucus in the early stages early em bryonic mortality and lowered semen quality There is circumstantial evidence that the disease can be carried through an apparently normal pregnancy and produces subsequent infertility The herd breeding problem usually resolves without treatment within 3–5 months after the beginning of the mating season No obvious associated ovarian abnor malities have been found

McClure showed that by intravaginal inoculation of the agent he could reproduce the condition in heifers However at the time of the latest report he had not studied the direct effect of the virus on fertility

The original report from the United States of a vaginitis associated with infertility of cattle was by Kendrick *et al* (1956) The condition involved both beef and dairy cattle and was first observed about 3 years ago in California While the clinical symptoms varied somewhat from one outbreak to another a vaginitis ranging from mild to severe and usually accompanied by a cervi citis was common to all cases The bulls in these herds rarely if ever showed clinical signs of infection although evidence incriminated some as transmitters of the disease

The condition is recognized clinically by the presence of a postcoital dis charge in cattle in herds which usually have a history of breeding problems Occasionally unbred heifers also show clinical evidence of infection but this observation has thus far been confined to herds containing affected cows On physical examination variable degrees of congestion of the vaginal mucosa frequently accompanied by congestion and hyperemia of the cervix are observed A yellowish gelantinous but tenacious exudate ranging in amounts from several milliliters to one hundred or more milliliters is found on the floor of the vagina The exudate is periodically discharged with the result that the tail and buttocks become soiled making it relatively easy to recognize affected animals The discharge persists for variable lengths of time in individual cows In some cases it ceases after a short time only to reappear at intervals In other outbreaks the course of the disease is relatively short in the individual animal without any tendency to recur There is no evidence of fever in affected cattle and the uterus is not involved

The herd picture varies considerably from one outbreak to another In some

cases only a few animals are involved, and these usually recover quickly In other herds the disease persists for long periods of time or at least appears periodically over a long period In such outbreaks the morbidity rate is some times as high as 50% In herds that undergo the more prolonged type of infection the symptoms are more marked and in one herd from which a virus was isolated retention of the placenta was a common feature

The conception rate is seemingly lowered although some animals conceive while the vaginitis exists However in the more severely affected herds a high percentage of the cows return for service and many of these do not conceive

EXPERIMENTAL STUDIES

Observations by the California investigators indicated that the condition was infectious since it could be transmitted from infected bulls to cows and vice versa by coitus Infected bulls as a rule showed no clinical evidence of infec tion One animal however exhibited a mild vesiculitis which at autopsy was found to involve thickening and fibrosis of the seminal vesicles and the presence of considerable amounts of pus therein

It was found that the infection could be readily transmitted by instilling either vaginal exudate or semen from infected animals into the vagina of virgin heifers thus demonstrating that the etiological agent or agents was present in these materials The fact that filtrates of vaginal exudates of in fected cows produced the condition as readily as unfiltered discharges not only eliminated the possibility that the condition was of bacterial etiology but definitely incriminated either a virus or other of the filterable agents as the cause

Shortly after ascertaining these facts a filterable agent was isolated in chicken embryos from vaginal discharges of an experimentally infected cow The agent was readily propagated in the embryo and regularly produced 100% mortality after several serial passages therein A second isolation was made in tissue culture of fetal bovine kidney cells from filtered exudates obtained from a field case of the disease This isolate could also be propagated in chicken embryos while the original isolate could be grown readily in tissue culture Efforts to propagate both agents in mediums used for the growth of pleuro pneumonia like organisms (PPLO) were unsuccessful indicating that the isolate was in each case a virus Great difficulty was experienced in adapting these viruses to suckling mouse brain and if adaptation occurred it was very incomplete Attempts to isolate virus from semen were unsuccessful

Each isolate when introduced as a chicken embryo culture into heifers by the vaginal route usually but not always produced a vaginitis that was grossly identical but milder than either the vaginitis which occurred under field conditions or the vaginitis produced experimentally by the inoculation of field materials Nevertheless since the virus was in each instance readily

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reisolated from the experimentally inoculated heifers it was assumed to be the cause of the infection that was produced

During these experiments an observation was made which suggested that the condition is transmitted not only by coitus but by other means as well This was revealed when in order to determine the effect of non infectious chicken embryo tissues on the genital tract of virgin heifers one heifer was inoculated intravaginally with a suspension of tissues from uninoculated embryonating hen eggs while two test animals received the egg propagated virus in the form of a chicken embryo suspension All three animals were placed in the same boxstall Within 48 hours the test animals exhibited the typical clinical signs of the infection On the eighth day however the control heifer also exhibited a vaginitis accompanied by the characteristic vaginal discharge Since a separate speculum was used for the examination of each animal and other precautions taken to avoid mechanical cross infection it was concluded that the condition must have been transmitted from the test heifers to the control by contact Later it was learned that the vaginitis observed in South Africa can also be transmitted by contact

To determine the relationship of the two California isolates cross serum virus neutralization tests were conducted in tube cultures of fetal bovine kidney cells using the serums of normal cattle and of cattle that had recovered from the natural and the experimentally produced infection Serial dilutions of each serum sample were mixed with a constant amount of virus and the mixtures incubated at room temperature Each dilution was then inoculated *into tubes of fetal bovine kidney cells and incubated for an appropriate length* of time The results of these tests established beyond a doubt that both virus isolates are identical

The senior author took advantage of a recent 6 month assignment in South Africa to observe field cases of vaginitis which still occur to some extent in that country and to make comparative serological studies between one of the California strains of virus and the South African isolate

During this study some rather interesting comparisons were noted between these two virus isolates Rather surprising was the finding that the South African virus could not be propagated in tissue cultures of beef kidney cells On the other hand the California virus could not be adapted to infant mice by intracerebral inoculation whereas the African virus adapted readily Both virus strains however were about equally pathogenic for chicken embryos

The test serums used for the comparative study were prepared by injecting rabbits intravenously with chicken embryo propagated cultures of each strain of virus After a series of injections the rabbits were bled and the serums were separated and inactivated at 56 C for 30 minutes The tests consisted essentially of titrating each virus strain in the presence of preinoculation serum and postinoculation homologous and heterologous antiserum using tissue culture infant mice and chicken embryos as the indicator systems The test mixtures

which consisted of decimal dilutions of virus in the presence of each serum in constant dilution were incubated for 75 minutes at room temperature and then inoculated to each of the indicator systems employed Readings were taken when the control series in each case showed the appropriate reaction

Since both strains of virus could be propagated in chicken embryos reciprocal cross tests were conducted in this host However only the cross test was conducted in tissue culture and in mouse brain since the California virus could not be propagated satisfactorily in the latter and the South African strain failed to multiply in tissue culture

The results of these tests demonstrated a complete lack of antigenic relationship between the California and the South African viruses in that insignificant amounts of each were neutralized by the heterologous antiserum whereas three to four logs of virus were neutralized in chicken embryo and mouse brain by the homologous serum in each case It was found that rabbit serum unfortunately contains nonspecific substances which neutralize the California virus in tissue culture but not in chicken embryo As a consequence the results of the *in vitro* cross test were not as well defined as those for which the other indicator systems were used Nevertheless a difference in titer which was regarded as significant was detected between the preinoculation samples and the postinoculation sample of the homologous test series but not of the heterologous series

Antiserums prepared in rabbits and guinea pigs for Millar s strain of virus were later obtained and cross neutralization tests conducted using the California virus as antigen The results of these tests were unfortunately inconclusive

Since rabbit serum has been found to neutralize the California strain of vaginitis virus nonspecifically while the serum of a high percentage of California cattle that were tested were found to contain antibodies for this virus it appears that both serums would be unsatisfactory for serological studies in tissue culture at least on possibly all strains of vaginitis virus Fortunately it was found that a serum with a highly potent neutralizing effect on the California strain can be produced in sheep Should sheep prove satisfactory for the production of antiserums for all strains of vaginitis virus it will then be possible to study all strains serologically on a comparable basis

DISCUSSION

It is apparent that similar perhaps identical syndromes characterized most consistently by a vaginitis are associated with infertility of cattle in South Africa England New Zealand and the United States However the role played by each in causing the infertility observed and their relationship to each other are still largely a matter of speculation Millar (1955) however has produced substantial evidence that the condition which occurs in dairy cattle in England definitely causes sterility in both male and female animals

The marked changes which result in the semen of infected bulls and the uterine pathology by which the disease is characterized in the female are ample evidence of this On the other hand the infectious syndromes occurring in South Africa and California apparently have no adverse effects on the quality of the semen and the infection in the cow is relatively mild and confined to the vagina and cervix It is difficult to decide from the available literature whether the vaginitis reported from New Zealand more closely resembles the condition described by Millar or the conditions which occur in South Africa and California McClure (1956 1957) who reported the occurrence of the disease in New Zealand refers to a lowered quality of the semen and early embryonic mortality but states that insufficient controlled experiments have been conducted to determine conclusively the effect of the condition on fertility

It would appear that the syndromes referred to above can be separated on a clinical basis into two groups Because of its distinctive effects in both male and female animals the condition occurring in England and possibly the one in New Zealand would logically comprise the first group The conditions reported from California and South Africa would come under the second group The mild form of vaginitis briefly described by Millar (1955) in England would also appear to belong in the second category

With the exception of the milder of the two diseases affecting cattle in England a virus has been isolated by much the same technique from each of the conditions described herein which might suggest that the same condition is involved in each case Limited serological studies have however indicated the likelihood of wide antigenic differences existing between the various strains of virus isolated While it has been determined that both strains of California origin are identical they not only differ serologically but in certain cultural respects as well from the virus recovered in South Africa Despite this fact on a clinical basis the condition in each country appears to be the same Comparative serological studies have not as yet included the virus isolates from England and New Zealand However in view of the findings referred to above it would seem not unlikely that these viruses will also be found to differ not only from each other but from the South African and the California strains as well Whether such a finding could be interpreted as evidence that a distinct disease is involved in each case remains to be determined by more precise means

In this connection it should be noted that the situation might be analogous to that of the bluetongue and the African horsesickness viruses These viruses exist as several antigenically distinct types which can be differentiated only by serum virus neutralization tests and in the case of bluetongue by cross protection tests in sheep also Owing to the broad antigenic specificity of the complement fixation test it can readily identify all strains of the particular virus but cannot distinguish between the different types With the application

of the complement fixation reaction or some other suitable test procedure to studies on the various vaginitis isolates it might be found that all are the same virus existing as a number of serologically distinct antigenic types

An observation that has caused some speculation on the part of the California workers at least is that vaginitis cannot be reproduced consistently with the California strain of virus and most of the experimentally produced cases are milder than those that occur under natural conditions The South African workers have likewise made this observation with their strain of virus This might indicate that the virus causes the infection only in concert with another agent or agents Millar (1955) in fact suggests that the virus which he isolated might cause infection indirectly by enhancing the virulence of the natural flora of the female genital tract or that more than one virus may be involved A more extreme view would be that the viruses which have been isolated from the mild cases of vaginitis at least belong to the so-called orphan group and as such play little or no role in the infections from which they have been recovered However under certain circumstances which are as yet unknown they either directly or indirectly give rise to infection

A second possible explanation for the mildness of the experimental infection and for its occasional failure to develop is the possibility that antibodies for the vaginitis virus are widespread among the cattle population In this event it would be impossible to avoid using immune or partially immune cattle in studies of the disease In fact a limited survey has shown that a high percentage of California cattle both young and old carry antibody titers for the California strain of vaginitis virus The correlation of these titers to resistance has not yet been determined

In the opinion of the authors the initial step in attempting to elucidate the vaginitis syndrome should be to determine the relationship to each other of the different virus strains that have been isolated from cases of vaginitis in each of the four countries where the disease has occurred While the geographical distribution of the condition imposes certain limitations on the scope of such a study we nevertheless hope to make a serological study in the near future on all known strains of the virus Field projects designed to determine the effect of the virus on fertility should also be carried out Such studies to be undertaken on the California strain of virus at Davis are also contemplated for the near future

SUMMARY

The salient clinical features of each of the vaginitis syndromes that have been reported from South Africa England New Zealand and the United States are described together with a summary of the experimental studies that have been conducted on each thus far

The possible effect of each entity on the fertility of cattle and their relationship to each other are discussed

Approaches that might help to elucidate the vaginitis syndrome are suggested

Acknowledgments The authors express their sincere gratitude for the extraordinarily fine assistance rendered by Miss E M Wada and Mr J K Saito throughout the course of the studies reported herein

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It is evident from Table I that approximately 50% of the infected rams examined were producing semen of a definitely inferior quality and that only 25% could be placed in a strongly fertile category

On a practical basis it should be pointed out that many of our rams are placed with the ewes during the months of May through August a period which includes very hot summer temperatures It seems reasonable to assume therefore that a ram whose fertility is already reduced by epididymitis and is then subjected to hot environmental temperatures is not doing his part in settling ewes

These observations lead to questioning of the validity of some of our established criteria for measuring the reproductive capacity of the ram

Concrete specific information regarding the effect of epididymitis on the fertility of the ram is rather limited but it is hoped that a more widespread interest in this disease will stimulate additional research

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NEOPLASMS OF THE GENITALIA OF THE BOVINE*

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MANY articles have been written on the various causes of bovine sterility but there appears to be very little information on neoplastic diseases of the bovine genitalia and their relationship to reproductive problems Perhaps this is partly due to the infrequent recognization of tumors of the internal genital organs in cattle As a consequence there are very few cases on which clinical observations have been recorded in cows afflicted with neoplastic disease of the internal genital organs The common practice of sending to market animals that are no longer profitable as breeders affords an opportunity to make an examination at the time of slaughter for evidence of disease conditions which may not have been recognized or suspected clinically As a rule no clinical history is available on such animals and correlation between the necropsy findings and the reason for the disposal of the animal is usually lacking Nevertheless the records of the Meat Inspection Division of the U S Department of Agriculture are of considerable value in that they often disclose a greater incidence of some diseases than has been previously supposed Tumors of food producing animals fall in this category and the cases submitted to Federal laboratories for microscopic diagnosis provide a fruitful source of information on neoplastic diseases

SOURCE OF MATERIAL

Monlux Anderson and Davis (1956) made a review of all cases of neoplastic disease of meat inspection specimens in the files of the Animal Disease and Parasite Research Division Laboratory of the U S Department of Agriculture at Denver Colorado Special attention was given to cases of adenocarcinoma of the uterus in the bovine and a rather significant number were found in the records The findings in this study disclosed 26 confirmed cases of primary uterine adenocarcinoma where the entire uterus was available for examination and 62 cases which were diagnosed as probable primary uterine adenocarcinoma based on the distribution and histologic similarity of the metastatic lesions to

* From the Animal Disease Research Laboratory Animal Disease and Parasite Research Division Agricultural Research Service U S Department of Agriculture Denver Colorado Dr Davis is now retired from the Agricultural Research Service

those observed in proved cases The total 88 cases reported is one of the largest series recorded judging from a rather comprehensive review of the literature

It was of interest that in all the cases of adenocarcinoma of the uterus the tumor had already spread to other parts of the body at the time of slaughter necessitating the rejection of the entire carcass as unfit for human consumption The regional lymph nodes draining the uterus the lungs and the thoracic lymph nodes were the common sites of metastases Tumor implants were also frequently seen on the peritoneal surfaces and occasional cases showed spread to the fallopian tube and ovary

It is regrettable that the breeding records of animals with uterine cancer in this series were not available for critical study To imply that adenocarcinoma of the uterus with resultant sterility was the reason for sending these animals to market may be presumptous however if one can accept the observations made at necropsy of routinely slaughtered animals as a criterion it is reasonable to make such an assumption Supporting evidence is the fact that of the 26 proved cases of uterine cancer in our series 13 were found to be nongravid at the time of slaughter The presence or absence of pregnancy was not recorded in the remaining cases In the few reported cases of pregnancy in cows affected with adenocarcinoma of the uterus there was no way of determining if conception occurred before or after the onset of the tumor One case of pregnancy in a cancerous uterus observed subsequent to this review is on record in the Denver laboratory files In this instance a ten year old cow was found at time of slaughter to have a typical adenocarcinoma in the midportion of one horn and a normal 2-3 month fetus in the other Widespread metastases were found in the lungs and in the bronchial sublumbar and internal iliac lymph nodes The carcass was condemned Monlux Anderson and Davis (1956) also cited the case of a 6 year old cow that had been manually delivered of a normal calf Three weeks post partum the animal became inappetent and constipated and exhibited difficulty in urinating A week later pelvic examination revealed a palpable tumor of the uterus near the cervix and greatly enlarged pelvic and sublumbar lymph nodes The animal became dyspneic and died 40 days after calving Death was attributed to massive uterine hemorrhages into the peritoneal cavity Upon microscopic examination the tumor proved to be a uterine adenocarcinoma with metastases to the sublumbar lymph nodes and lungs

In further studies on the relative occurrence of neoplastic diseases of food producing animals Monlux Anderson and Davis (1956) made a survey of all bovine ovine and porcine tumors encountered in 1953 and 1954 in the Denver abattoirs operating under Federal inspection During the 2 year period 280 neoplasms exclusive of the common eye tumor in the bovine were collected for gross and microscopic examination The specimens were obtained to establish their identity for evaluation of the common tumors which might be encountered on post mortem inspection

The 280 neoplasms consisted of 186 in cattle 66 in sheep and 28 in swine For the purposes of this paper only those cattle tumors affecting the genital organs will be considered A total of 45 of the 186 bovine specimens or roughly 24% showed involvement of the genitalia The type of neoplasm and the organs involved were further classified as follows Adenocarcinoma of the uterus 26 cases malignant lymphoma (lymphosarcoma) of the uterus 6 leiomyoma of the uterus 4 granulosa cell tumor of the ovary 6 cystadeno carcinoma of the ovary with metastases 1 squamous carcinoma of the vulva 1 and fibroma of the penis 1 case It should be mentioned that the 26 uterine adenocarcinomas recorded in this survey were included among the 88 cases already discussed and do not represent additional cases

No cases of carcinoma of the cervix or vagina were encountered during the 2 year survey There is however a record of two cervical cancers and one of the vagina in the files of the Denver laboratory These are only mentioned here because of their infrequent occurrence and will not be discussed further

The observations on epithelial tumors of the eye or it appendages collected during this survey were reported separately by Monlux Anderson and Davis (1957) The results of their study of 722 ocular squamous cell tumors indicate that neoplasms of the genitalia seldom result from metastasis of cancer eye in cattle

It is of interest that no examples of tumors of the testes or prostate gland were observed in the 2 year period or for that matter at any time in the Denver laboratory They are probably rare in the bull Tumors of the penis in the bull are of rather frequent occurrence and are most commonly seen in younger animals The fact that fibroma of this organ was encountered only once in our 2 year survey is evidence of the fallacy of trying to arrive at the relative occurrence of animal neoplasms from the records of slaughtering establishments alone

An example of each of the types of tumors of the bovine genitalia encountered in this survey has been selected for illustration of their gross and microscopic features

PATHOLOGICAL CONSIDERATIONS

Adenocarcinoma of the Uterus

Uterine adenocarcinomas usually arise from the glandular tissue of the endometrium They may occur in either cornu or the body of the uterus and more often develop as single growths One case in our collection showed three distinct tumor masses two in one cornu and one in the other In another instance two separate growths were found in one cornu A rather common finding was the tendency of the tumors to form annular constrictions of the involved areas The following example typifies the gross and microscopic features of this tumor The subject was an 8 year old nonpregnant cow which at necropsy revealed a 5 × 3 × 2 cm growth in the midportion of the left

those observed in proved cases The total 88 cases reported is one of the largest series recorded judging from a rather comprehensive review of the literature

It was of interest that in all the cases of adenocarcinoma of the uterus the tumor had already spread to other parts of the body at the time of slaughter necessitating the rejection of the entire carcass as unfit for human consumption The regional lymph nodes draining the uterus the lungs, and the thoracic lymph nodes were the common sites of metastases Tumor implants were also frequently seen on the peritoneal surfaces and occasional cases showed spread to the fallopian tube and ovary

It is regrettable that the breeding records of animals with uterine cancer in this series were not available for critical study To imply that adenocarcinoma of the uterus with resultant sterility was the reason for sending these animals to market may be presumptuous however if one can accept the observations made at necropsy of routinely slaughtered animals as a criterion it is reasonable to make such an assumption Supporting evidence is the fact that of the 26 proved cases of uterine cancer in our series 13 were found to be nongravid at the time of slaughter The presence or absence of pregnancy was not recorded in the remaining cases In the few reported cases of pregnancy in cows affected with adenocarcinoma of the uterus there was no way of determining if conception occurred before or after the onset of the tumor One case of pregnancy in a cancerous uterus observed subsequent to this review is on record in the Denver laboratory files In this instance a ten year old cow was found at time of slaughter to have a typical adenocarcinoma in the midportion of one horn and a normal 2-3 month fetus in the other Widespread metastases were found in the lungs and in the bronchial sublumbar and internal iliac lymph nodes The carcass was condemned Monlux Anderson and Davis (1956) also cited the case of a 6 year old cow that had been manually delivered of a normal calf Three weeks post partum the animal became inappetent and constipated and exhibited difficulty in urinating A week later pelvic examination revealed a palpable tumor of the uterus near the cervix and greatly enlarged pelvic and sublumbar lymph nodes The animal became dyspneic and died 40 days after calving Death was attributed to massive uterine hemorrhages into the peritoneal cavity Upon microscopic examination the tumor proved to be a uterine adenocarcinoma with metastases to the sublumbar lymph nodes and lungs

In further studies on the relative occurrence of neoplastic diseases of food producing animals Monlux Anderson and Davis (1956) made a survey of all bovine ovine and porcine tumors encountered in 1953 and 1954 in the Denver abattoirs operating under Federal inspection During the 2 year period 280 neoplasms exclusive of the common eye tumor in the bovine were collected for gross and microscopic examination The specimens were obtained to establish their identity for evaluation of the common tumors which might be encountered on post mortem inspection

In this case the animal was carrying a 3 month fetus in the presence of a 40 lb tumor The case record did not indicate whether the involved or normal cornu was gravid The largest uterine leiomyoma in our collection weighed 50 lb The opposite cornu also contained a 6 lb tumor The uterus was non-gravid

Microscopically the tumors are composed of interlacing bundles of smooth muscle cells containing elongated granular nuclei with rounded ends (FIG 5) In areas of the growth where interweaving of the muscle bundles is less marked the nuclei are laid down in an orderly parallel fashion and are of uniform size and shape Mitoses are not usually observed The sausage shaped appearance of the nuclei aids in the differentiation from the more spindled nuclei of the fibroblasts A variable amount of fibrous connective tissue is interspersed throughout the tumor which contributes to the resilience of the gross lesion Areas of necrosis hemorrhage and cystic degeneration are frequently seen in the larger neoplasms

Malignant Lymphoma of the Uterus

The lymphoid tumor of the bovine is of common occurrence and may arise in any part of the body where lymphoid tissue is normally present This neoplasm is invariably fatal if permitted to run its natural course It is one of the most frequent causes for condemnation of entire carcasses for neoplastic disease in meat inspection The malignant lymphoma usually grows and spreads rapidly and may involve any of the tissues or organs of the body The uterus is no exception since among the 20 cases of this neoplasm encountered in our survey multiple uterine lesions were found in 6 in association with other anatomic sites of involvement (FIG 6) Additional cases studied in the Denver laboratory showed solitary lymphoid tumors in the uterus of which one actually weighed 40 lb In other instances we have observed diffuse thickening of the entire uterus with neoplastic lymphoid tissue One case was of particular interest in that a 5 year old cow showing extensive involvement of the uterus was carrying a nearly full term calf The internal iliac lymph nodes and the auricle of the heart were also tumefied We also have observed a 4–5 month old normal fetus in a tumefied uterus Here again there was no way of determining if conception occurred before or after the onset of the neoplastic process

The malignant lymphoma is usually grayish white compact and of soft consistency with a minimal supporting connective tissue stroma In some of the larger growths however the tumor may contain strands of connective tissue separating the neoplastic mass into lobules Also the larger lesions not infrequently contain areas of hemorrhage and necrosis The macroscopic features of malignant lymphoma aid in the differentiation from the yellowish cirrhotic lesion of carcinoma and the flesh pink firm resilient lesion of the leiomyoma

cornu of the uterus The mass had produced an annular constriction with almost complete occlusion of the lumen On cut section the growth was yellowish and sclerotic (FIGS 1 and 2) The tumor had spread to the left ovary and broad ligament adjacent to the involved cornu In addition there were metastases to the peritoneal surfaces the iliac sublumbar, and portal lymph nodes in the abdominal cavity and to the lung bronchial, and mediastinal lymph nodes in the thoracic cavity

Microscopically the tumor was made up of low columnar epithelium arranged in a rather characteristic glandular pattern although in some areas, the tumor cells grew in solid nests (FIG 3) Mitoses were common An abundant fibrous stroma was interspersed throughout the growth which accounts for the sclerotic nature of the tumor The neoplastic cells had invaded extensively both the muscularis and endometrium but there was no apparent break through either the serosal or endometrial surfaces There was however lateral spread of the tumor cells under an intact endometrial lining The newly formed glandlike structures frequently contained cellular debris The glandular pattern of growth together with an abundant fibrous stroma was maintained in all the metastatic lesions It was this characteristic microscopic picture together with the rather common distribution of the metastases in known cases of primary uterine adenocarcinoma that enabled us to diagnose many cases of probable primary adenocarcinoma of the uterus when that organ was not available for examination

Leiomyoma of the Uterus

Leiomyomas not infrequently have their origin in the smooth musculature of the genital tract but the uterus of cattle seems to show a greater predilection for this type of tumorous growth They commonly contain a relatively large amount of fibrous connective tissue in conjunction with the neoplastic smooth muscle cells and are also referred to as uterine fibroids or fibromyomas These tumors are usually benign and remain in the uterine wall even though they may grow to massive proportions They are confined to the muscular wall by a fibrous capsule and grow by expansion The tumors may be single or multiple, involving either the body or the horns of the uterus The four cases encountered in this series were solitary growths the largest measuring 28 × 18 × 7 cm Two of the animals were pregnant at the time of slaughter One contained a nearly full term calf in a cornu despite the presence of a 11 × 10 × 6 cm leiomyoma in its muscular wall The other had a tumor 20 cm in diameter in a nongravid cornu while carrying a 2–3 month normal fetus in the opposite cornu (FIG 4) Whether the first animal could have delivered normally in the presence of the tumor is problematic Suffice to say pregnancy may be interfered with if the body of the uterus is affected or if both cornua contain multiple tumors Among 11 additional leiomyomas of the bovine uterus on record in the Denver files, only one was associated with pregnancy



FIG 1 Adenocarcinoma of the uterus bovine One horn contains a neoplastic mass producing a complete annular constriction (arrows) The corresponding ovary is tumefied From *Am J Vet Res* (January 1956) 17 53

In histologic section the tumor consists of lymphoid cells growing in a compact but unrestricted manner (FIG 7) The cells are irregularly spherical with hyperchromatic oval nuclei and a minimum of cytoplasm The neoplastic cells may show considerable variation in size, particularly in the more rapidly growing tumors Mitosis is usually demonstrable without difficulty The tumor may grow in nodular masses or proliferate in a diffuse manner, with obliteration of the invaded tissues

Granulosa Cell Tumor of the Ovary

Primary ovarian tumors in cattle are by no means rare Their observance in animals coming to slaughter constitutes the principal source of information on this entity Our survey revealed 6 granulosa-cell tumors among 42 neoplasms affecting the internal genital organs in the bovine These and an additional 19 cases in the Denver collection were classified as granulosa-cell tumors on the basis of their histologic appearance Clinical data were lacking in all but one animal and this particular case was made the subject of a separate report by Kingman and Davis (1940) Associated pregnancy was not recorded in any of our cases but this does not preclude such a possibility Data on this aspect would have been of extreme importance in evaluating the relationship of this tumor to reproductive interference

Granulosa-cell tumors are seen in various stages of development at necropsy and we have observed massive growths weighing 20 lb or more They are usually ovoid have a smooth outer surface and may be more or less lobulated The cut surfaces may be grayish or yellow and have a granular appearance The tumor is usually soft and quite vascular Broad bands of connective tissue often intersperse the growth The larger lesions may contain many cysts diffuse areas of hemorrhage and necrosis (FIG 8) They can arise in either ovary but seldom occur bilaterally A few of our cases showed numerous tumor implants on the abdominal serosa resulting either from spillage of neoplastic cells from a ruptured lesion or by transperitoneal metastasis Associated metastases were also observed in the internal iliac or mediastinal lymph nodes in 5 cases

Langham and Clark (1945) and Kingman and Davis (1940) have shown that the tumor can provoke a remarkable feminizing hormonal influence with resultant symptoms indicative of nymphomania Cystic ovaries can also produce a similar syndrome and differentiation must be made by pelvic examination If an ovarian tumor is suspected it can be removed surgically and if the removal is done in time the animal may return to normal It should be mentioned that the tumor can be mistaken for pregnancy based on the presence of a palpable mass in the pelvic cavity This happened in the two cases of granulosa-cell tumor reported by the above authors in which the error in diagnosis was not discovered until the animals were autopsied

Microscopically the granulosa-cell tumor may present an extremely variable



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FIG 4 Leiomyoma of the uterus bovine Incised tumor [illegible]0 cm in diameter in the wall of the right horn The opposite horn carried a 2 3 month live fetus



FIG 5 Leiomyoma of the uterus bovine Section shows interlacing bundles of smooth muscle cells and areas of fibrous connective tissue Hematoxylin–eosin 110

FIG 2 Adenocarcinoma of the uterus bovine Cut surface of neoplasm shown in Fig 1 Note enlarged tumefied ovary at right as compared with uninvolved ovary at left From *Am J Vet Res* (January 1956) **17** 53

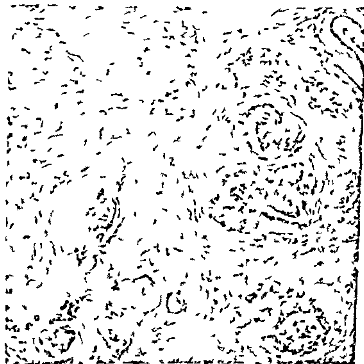


FIG 3 Adenocarcinoma of the uterus bovine The section shows neoplastic glandlike formations resting in an abundant fibrous stroma Note cellular debris within the lumens Hematoxylin–eosin ×110

Fig. 8. Granulosa cell tumor of the ovary, bovine. Tumor of right ovary size of indoor baseball. The growth was multilocular and extremely hemorrhagic. Numerous implants were present on visceral and parietal peritoneum. Note the serosal implants on the opposite ovary.

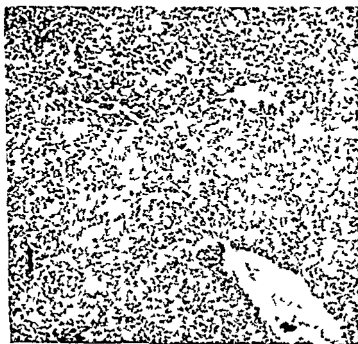


Fig. 9. Granulosa cell tumor of the ovary, bovine. Section shows a compact, highly cellular pattern of growth. Note small area of cystic degeneration, lower left. Hematoxylin–eosin. ×110.

FIG 6 Malignant lymphoma of the uterus bovine Multiple subserous nodular growths in the body and both horns Associated lesions were present in the mediastinum liver spleen kidney and the portal mesenteric and sublumbar lymph nodes

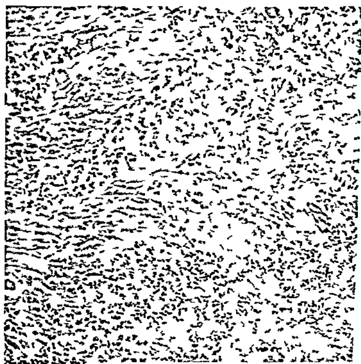


FIG 7 Malignant lymphoma of the uterus bovine Section shows lymphoid tumor cells invading the wall of the uterus Hematoxylin–eosin 110

Fig 12 Fibroma of the penis bovine Irregular oval lobulated tumor (15 10 5 cm) completely enveloping the glans penis Note the extensive surface ulceration of the distal two-thirds of the lesion A separate lesion 1 cm in diameter is below the main mass and a similar growth 5 cm in diameter is attached to the prepuce adjacent to the large tumor mass

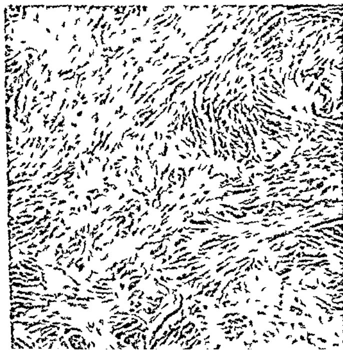


FIG 10 Squamous-cell carcinoma of the vulva bovine The cancerous growth nearly envelopes the entire external orifice of the vulva



FIG 11 Squamous cell carcinoma of the vulva bovine Section shows large neoplastic cells separated into irregular nests by connective tissue stroma Note the cellular infiltrate in the stromal tissue Hematoxylin eosin ×110

pattern in different cases and within individual tumors Usually the cells have a morphologic resemblance to granulosa cells which is the most important criterion for diagnosis Sometimes the polyhedral tumor cells become spindled to produce a sarcoma like pattern These are believed to be transitions from the more typical epithelial areas Other areas may show a cylindroid or adenomatous arrangement of the cells depending to some extent on the amount and manner of distribution of the supporting connective tissue stroma Again the tumor cells may grow in diffuse masses with little or no suggestion of a folliculoid pattern (FIG 9) The degree of mitosis is variable and may be scant in some cases and abundant in others Degenerative changes are commonly present in the larger tumors

Carcinoma of the Vulva

Squamous-cell carcinoma of the vulva is rarely observed and is only of importance where a valuable breeding animal may be concerned Early lesions may not be recognized as cancerous growths and the five examples in our files were fairly well advanced when submitted for histologic examination These presented a rugose type of lesion with considerable surface ulceration and secondary infection On section the cut surface appeared as a gray or yellowish granular lesion The tumors probably had their origin at the mucocutaneous junction of the labium and involved a part or nearly all of the external vulvar orifice (FIG 10) In each instance the tumor had invaded the underlying subcutaneous and muscle tissue It is conceivable that in advanced cases there could be mechanical interference with breeding or perhaps reluctance on the part of the cow to accept the service of the bull

Histologically the tumor is made up of irregularly shaped compact nests of squamous epithelial cells separated by a variable amount of vascular fibrous stroma (FIG 11) The tumor spreads to the adjacent normal tissues by infiltration of fingerlike strands of neoplastic cells The supporting stromal tissue frequently contains lymphocytes and eosinophiles Rapidity of growth may result in areas of necrobiosis due to inadequate blood supply In some of the epithelial nests the older cells in the center become cornified and stain more acidophilic and their nuclei are arranged in a concentric fashion These formations are frequently referred to as epithelial pearls and often characterize the squamous-cell type of carcinoma

Fibroma of the Penis

Tumors of the penis of the bull are common and are frequently seen in younger animals according to McEntee (1950) These growths are fibrous in nature and are clinically benign even though they may attain considerable size and occasionally show some histologic evidence of malignancy They seldom recur following complete excision and to our knowledge are not known to

SUMMARY

In a series of 186 cases of neoplasia in the bovine 24% showed involvement of the genitalia

In cattle primary adenocarcinoma of the uterus has recently been shown to be one of the more frequently occurring tumors in the female and fibroma of the penis is the most common genital neoplasm in the male

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MCENTEE, K (1950) Fibropapillomas of the external genitalia of cattle *Cornell Vet* 40 304

invade locally or metastasize The lesions are usually single and may arise on the glans penis or other areas on the body of the organ Lesions can also occur on the sheath Multiple growths occur, but less frequently (FIG 12) They often have a cauliflower appearance The tumor may interfere with copulation or there may be hesitancy or refusal of the bull to serve Their size often prevents retraction of the penis and the tumor is subject to trauma hemorrhage, and ulceration The cut surface shows a grayish white firm glistening type of tissue

A similar type of fibrous tumor of the vulva and vagina of heifers has been observed They may grow to sufficient size to protrude from the vulva before parturition but more often are first seen at calving time The true nature of these tumors in heifers and young bulls has not been determined but because of their simultaneous occurrence in several animals stabled or running together some authors McEntee (1950) suspect an infectious etiology perhaps of the nature of a virus

Microscopically the tumor of the penis is composed of fibroblasts arranged in interlacing bundles (FIG 13) A variable amount of collagen is formed, which increases with the age of the tumor In the majority of cases the neoplastic cells resemble the mature fibroblast but other tumors contain areas in which the cells show a somewhat disorderly arrangement their nuclei are irregular in size and shape and mitotic activity is apparent These less orderly areas are at least suggestive of histologic malignancy and such tumors have been diagnosed by some pathologists as fibrosarcoma The covering epithelium of the tumor may show proliferation with papillary projections extending into the tumor substance The proliferating epithelium is considered a component part of the neoplastic process by McEntee (1950) who uses the term fibropapilloma to designate this tumor

DISCUSSION

Because of their relatively infrequent occurrence tumors of the genitalia do not represent a major economic factor in the over all problem of infertility in cattle Nevertheless they may be of sufficient importance particularly in valuable breeding stock to warrant consideration by the clinician in the differential diagnosis of the causes of infertility Furthermore it is probable that careful post mortem examination particularly at the abattoir will result in the recognition of neoplasia of the internal genital organs in many instances where the cause of infertility might otherwise remain unexplained

Except for removal of the readily accessible fibroma of the penis in the male surgical or other treatment probably has only limited application in the bovine Early diagnosis of neoplasia of the internal genital organs in the female will permit disposal of the animal for salvage and avoid the maintenance of an unprofitable animal in a futile attempt to obtain more calves

C L. DAVIS Dr Gassner in this article to which I previously referred the age was given for each of the 26 cases in which we were able to examine the uterus to determine the primary origin I believe the youngest animal was 3 years old and from there on up to 8 10 and 12 years of age I haven t seen it in a very young animal Now the second question you ask is most important and is one of the reasons why I was glad to have an opportunity to come up and present this problem at the Symposium We know very little about the clinical history of these animals with tumors of the bovine genitalia Our object in presenting this paper is to try to stimulate a little more interest in the diagnosis or the differentiation of these different types of sterility There is a lot we do not know about the causes of sterility and here we have come up with another one and very little is known about it There are very few instances in which clinical records were made on these cases and the reasons for it is that they are not detected by the clinician I have interviewed several of our leading veterinary clinicians in the Denver area and when I asked them what information they could give me on carcinoma of the uterus they knew nothing about it If we can get these clinicians and veterinarians to recognize the disease and give us something about the case histories then we will know more about this

F X GASSNER Thank you Dr Davis One more question please Have you any information on the anatomical status of the ovary in cases where you found adenocarcinoma?

C L DAVIS Dr Gassner here again practically all of our material was obtained from animals coming to slaughter We know nothing about them We don t know whether they had had veterinary examination or not We are assuming that these cattle were sent in because they were unable to become impregnated Two of the cases one in our own series and one that was reported in the foreign literature showed that there was pregnancy associated with this tumor but there was no way of telling whether the tumor occurred before or after conception Nothing was said about the condition of the ovary In all cases we had an opportunity to examine the animal but we felt that unless the ovaries were involved in a metastatic process that they were essentially normal

H T GIER Mr Chairman in our work on the uterus of the cow we have found in some 3 or 4 / of the uteri rather large fibroid cysts very definitely of bacterial origin Have you found any correlation between such cysts and carcinoma?

C L DAVIS Dr Gier by the time we get such cysts the boys on the killing floor usually have cut into them If they were abscessed we didn t get them because we were interested in neoplasia. I can t answer with any certainty but are you asking me if an abscess is a forerunner to carcinoma? If you are I say I don t think so At least if we can draw a correlation between what we see in the bovine and what we see in the human I have never read where an abscess of an ovary or a uterus or of any other organ precedes the formation of cancer cells

RUE JENSEN Thank you Dr Davis The next question is directed to Dr McGowan

KENNETH MCENTEE Has an attempt been made to infect bulls with the organism which causes epididymitis in rams?

BLAINE MCGOWAN To my knowledge no attempt has been made using the ram epididymitis organism in bulls There are however plans to attempt that in the future

D H MCWADE What has been found to be the effect of high scrotal temperature on conception rate in the valley ewes?

DISCUSSION

Monday July 1 1957

Afternoon Session

RUE JENSEN Presiding

DISEASES OF REPRODUCTION

RUE JENSEN As we proceed with the Panel this afternoon you are invited to make further comments on the questions if you wish

Members of the Panel on Reproductive Diseases are Dr Kenneth McEntee of Cornell University Dr C L Davis of U S D A who has just discussed Neoplasia of the Bovine Genitalia Dr D G McKercher from the University of California and his colleague Dr Blaine McGowan also from the University of California Dr V A Miller a member of the staff of this University and Mr Malcolm Trueblood from the Department of Bacteriology at the University of Wyoming I think we should start with questions directed to Dr Davis since his presentation is fresh in your mind

E W JONES What is the percentage incidence of adenocarcinoma of the bovine uterus in relation to the total number of cows slaughtered in the series reported?

C L DAVIS In a previous computation dealing with the diagnosis of adeno carcinoma published in 1956 in the Veterinary Research Journal we gave the total number of cattle slaughtered for that 2 year period but we did not attempt to calculate the percentage I might say that it is a small fraction of 1 / It was not my intention to say that carcinoma of the uterus is common but it certainly is far more common that one would expect from the information one gets from the literature or from our veterinary textbooks

RUE JENSEN Are there any comments from the floor on that subject If not Dr Davis another question is as follows Was any breed difference in susceptibility observed for adenocarcinoma of the uterus? I believe you indicated that no such difference existed for the malignant lymphoma

C L DAVIS In relation to adenocarcinoma I might say that our 2 year survey was conducted at the Federal establishment in Denver Colorado where 85–90 / of the cattle slaughtered are of the Hereford breed In our series where the breed was identified they were all White faced except one that animal was a Brown Swiss Reports of adenocarcinoma of the uterus made in foreign countries and in one or two instances in this country involved breeds other than the White faced Now if there are any White faced breeders here please do not accuse me of saying that the White face is more susceptible to adenocarcinoma of the uterus than other breeds It just so happened in our observation

F X GASSNER *My question Dr Davis concerns the relationship of age of the* bovine female and the general reproductive status of that cow to the incidence of carcinoma of the uterus Have you found that there is any relationship to recent calving or the stage of milk secretion? I wonder whether the incidence of uterine carcinoma is higher in older cows or do you ever find such neoplasm in cows below 5 years of age? In human medicine such relationship to advanced age exists

floor on the subject of epididymitis? If not let us proceed to the subject of Vibriosis in Sheep This question is directed to Dr Miller

E. M SACCHI Would you please elaborate on the size and type of inoculum used in oral inoculation of pregnant ewes?

V A MILLER Fifty grams of ground viscera from aborted lambs infected with *Vibrio fetus* was given orally to each ewe at the beginning of the 5th month of gestation

E M SACCHI Have you shown that oral infection is probably the route by which sheep become infected naturally?

V A MILLER Our work has all been done on an experimental basis From our findings resulting from oral inoculation of susceptible pregnant ewes in advanced stages of gestation and by the use of rams from infected flocks or artificially infected rams we consider oral infection of the ewe to be the route by which sheep become infected naturally

RUE JENSEN I believe that investigators at Montana State College have shown natural transmission by placing normal susceptible ewes in a pen which contained aborting ewes The next question is also to Dr Miller

C F HAWKINS Were the aborted lambs expelled previous to normal parturition or were they born dead at full term?

V A MILLER The majority of lambs were born dead approximately at full term There were a few that were born in an excessive state of weakness and lived up to two days following birth

C F HAWKINS Was infected bovine tissue used in any attempt to transmit vibriosis to sheep?

V A MILLER Our work was primarily with the infected tissue of lambs In work of other investigators bovine vibriosis was attempted to be passed to sheep in some of the earlier investigations I think it was only one ewe in which abortion was caused by bovine tissues However I am not sure whether the methods we are fol lowing today are the same

RUE JENSEN Dr McEntee would you care to speak on the question

KENNETH MCENTEE I don t as far as cattle are concerned I don t know of any successful attempts of producing vibriosis in cattle with the ovine strain

RUE JENSEN One experiment has been conducted in which six pregnant cows were inoculated orally with the same infected sheep tissues as were used to produce abortion in ewes without producing the disease in the cows The reverse procedure has also been done A few years ago we fed infected tissues from an aborted calf infected with *Vibrio fetus* to pregnant ewes without causing any evidence of the disease

J C OSBORNE How many days after oral inoculation did abortion occur?

V A MILLER The earliest abortions resulting after feeding infected tissue to susceptible ewes at the beginning of the fifth month of gestation was 4 days The mean was 13 2 with a standard error of 4 2

J C OSBORNE What was the route interval and amount of vaccine used in immunity studies?

V A MILLER All vaccines were given in 5 ml amounts intramuscularly with the ex eption of formalized infected tissue That was given subcutaneously All vaccina

BLAINE MCGOWAN I believe that question refers to the portion of the paper this morning comparing lambing percentage of one area of the state to another I personally have not done any experimental work on the effect of high temperature on reducing fertility in the ram Gunn in 1942 published a very lengthy article part of which dealt with the adverse effect of high temperature on fertility in the ram My recollection is that a range of 95 –105 F environmental temperature would adversely affect sperm potency and number This reduced fertility would be manifested in 2 or 3 weeks following exposure to increased environmental temperatures and would last for a period of 2–4 months It is considered a transient effect only because of the fact that eventually they did recover There was considerable variation from ram to ram

A M SORENSEN Can testicular biopsy be an aid to diagnosis of epididymitis? Some epididymides are very firm normally Is increased temperature of the scrotum measurable?

BLAINE MCGOWAN In answer to that first question yes I think you could use the biopsy method as an aid in the diagnosis of epididymitis Off hand however I fail to see as to what practical application that would have Palpation of the head or the tail of the epididymis itself will reveal those lesions It is true that an occasional epididymis will be slightly firmer than you might expect normally but it still does not have the feel that a truly infected epididymis will have We have been fooled occasionally by rams having an abnormally thick epididymal tunic We learned to distinguish between cases we were calling infected and which were shown upon subsequent slaughter not to be infected and the truly diseased glands In answer to the second question yes one can record the increased scrotal temperature on at least an artificially infected ram with epididymitis In many instances the disease is reflected in an increase systemic temperature as well Many of these animals carry a fever particularly in experimental cases and will also evidence some inappetence Whether field cases are less severe or whether the rams are less carefully examined I don t know but we have never had a call to see a ram sick with a natural case of epididymitis

STANLEY MUSGRAVE Does electroejaculation cause any alteration in ram semen and if so can those effects be separated from the effects of infection?

BLAINE MCGOWAN I can answer that question only indirectly It is our assumption that there would be no abnormal effects seen in semen collected by the Marden electroejaculator in the ram as compared to collection done with the artificial vagina There is some work currently being prepared for publication comparing bovine semen collected with an electronic ejaculator and with the artificial vagina There are some minor differences These differences that we see in these rams infected with this organism however are much more severe and of a nature which we certainly would not expect to be caused by the method of collection As a comparison semen values of our five control rams were within the normal range as set up by other methods of collection I would say that the differences we got were due to the disease and not to the method of collection.

RUE JENSEN In the publication of Dr Gunn in 1942 a large sample of rams was studied and the testicular lesions were classified As I recall the figures Dr Gunn found approximately 2 / of the effected rams showing varicocele of the spermatic cord The question is have you encountered varicoceles in rams in your study?

BLAINE MCGOWAN We have not found the varicocele in the spermatic cord

RUE JENSEN Thank you Dr McGowan Are there any further questions from the

floor on the subject of epididymitis? If not let us proceed to the subject of Vibriosis in Sheep This question is directed to Dr Miller

E. M SACCHI Would you please elaborate on the size and type of inoculum used in oral inoculation of pregnant ewes?

V A MILLER Fifty grams of ground viscera from aborted lambs infected with *Vibrio fetus* was given orally to each ewe at the beginning of the 5th month of gestation

E M SACCHI Have you shown that oral infection is probably the route by which sheep become infected naturally?

V A MILLER Our work has all been done on an experimental basis From our findings resulting from oral inoculation of susceptible pregnant ewes in advanced stages of gestation and by the use of rams from infected flocks or artificially infected rams we consider oral infection of the ewe to be the route by which sheep become infected naturally

RUE JENSEN I believe that investigators at Montana State College have shown natural transmission by placing normal susceptible ewes in a pen which contained aborting ewes The next question is also to Dr Miller

C F HAWKINS Were the aborted lambs expelled previous to normal parturition or were they born dead at full term?

V A MILLER The majority of lambs were born dead approximately at full term There were a few that were born in an excessive state of weakness and lived up to two days following birth

C. F HAWKINS Was infected bovine tissue used in any attempt to transmit vibriosis to sheep?

V A MILLER Our work was primarily with the infected tissue of lambs In work of other investigators bovine vibriosis was attempted to be passed to sheep in some of the earlier investigations I think it was only one ewe in which abortion was caused by bovine tissues However I am not sure whether the methods we are fol lowing today are the same

RUE JENSEN Dr McEntee would you care to speak on the question

KENNETH MCENTEE I don t as far as cattle are concerned I don t know of any successful attempts of producing vibriosis in cattle with the ovine strain

RUE JENSEN One experiment has been conducted in which six pregnant cows were inoculated orally with the same infected sheep tissues as were used to produce abortion in ewes without producing the disease in the cows The reverse procedure has also been done A few years ago we fed infected tissues from an aborted calf infected with *Vibrio fetus* to pregnant ewes without causing any evidence of the disease

J C OSBORNE How many days after oral inoculation did abortion occur?

V A MILLER The earliest abortions resulting after feeding infected tissue to susceptible ewes at the beginning of the fifth month of gestation was 4 days The mean was 13 2 with a standard error of 4 2

J C OSBORNE What was the route interval and amount of vaccine used in immunity studies?

V A MILLER All vaccines were given in 5 ml amounts intramuscularly with the exception of formalized infected tissue That was given subcutaneously All vaccina

tions were made approximately 1 month prior to breeding All animals received only one vaccination

RUE JENSEN The next question is directed to Mr Trueblood What difference *in vitro* exists between *Vibrio fetus* and the so-called *Vibrio dubius* besides the catalase and hydrogen sulfide reaction?

MALCOLM TRUEBLOOD At the regional Vibriosis meetings we classify *Vibrio* only on catalase and hydrogen sulfide activity The Wyoming station presented evidence at the 1956 regional meeting that showed some morphological differences Ristic Herzberg and Sanders (1956 Colonial and antigenic variations of *Vibrio fetus Am J Vet Res* **17** 803) of Florida have shown that there are colonial differences in growth between smooth rough and mucoid Outside of these few isolated and unrelated examples of differences I would hesitate to say there was any difference between the two vibrio other than the biochemical tests I might say that Dr Frank who is one of the promoters of the catalase test bases his test on the source from which the vibrio is isolated and perhaps if you went on that basis then you could say the source from which it is isolated is a difference Dr Frank indicated to me that the catalase negative are not found in the aborted fetus but only in the bull and the cow that would tend to show that there must be a site of location difference

RUE JENSEN The next question is to Dr Miller

M A BROWN How does the appearance of the placenta especially the cotyledons and surrounding area in case of vibriosis compare with changes produced by other types of infection?

V A MILLER In other cases of infection in pregnant ewes the placenta will also show hemorrhagic areas in the inter-cotyledonary areas and also within the cotyledon According to the experiences we have had with this if there is a mixed infection there appears to be a more hemorrhagic type of exudate present. Otherwise the identification would be based primarily on stained smear preparations from infected cotyledons in determining the morphology of the organisms or through histological studies of the membranes

M A BROWN Can the gross appearance be used as a diagnostic aid?

V A MILLER With certain limitations and I think that it has been explained that other bacterial infections will also produce lesions

RUE JENSEN Dr Miller would you comment on the gross lesions observed in some aborted fetuses in case of vibriosis of sheep?

V A MILLER Gross lesions vary We have found in some of the earlier abortions a blood tinged fluid is present in the abdominal and thoracic cavities Frequently subcapsular hemorrhages are present in the kidneys As the infection progresses the stomach contents become blood tinged thick and contain flaky material In lambs which live two days liver lesions were found Lesions may be several centimeters or larger in diameter some lesions can be identified only microscopically Frequently there is edema in the umbilical region

RUE JENSEN I believe that the consensus of opinion is that gross appearance of membranes is of little diagnostic value in vibriosis of sheep However in this area possibly in this country I know of no other disease of sheep that produces the high incidence of abortion that is often found in vibriosis Now that is not true in some other countries

The next question is directed to Dr Miller

R M MELAMPY Were any histochemical studies made on the placental tissues of infected ewes?

V A MILLER No

RUE JENSEN Mr Trueblood has Wyoming conducted any histochemical studies on infected placentas?

MALCOLM TRUEBLOOD None have been made as far as I know

RUE JENSEN The next question is to Dr Miller

M H EHLERS Were the tissues used to feed the yearlings subjected to a storage period between time of collection and administration?

V A MILLER The tissues were stored for several weeks at deep refrigeration which was at −50 C until time of use

RUE JENSEN Those tissues were the infected placenta and viscera from the infected aborted lambs As Dr Miller said they were stored in refrigeration for several weeks before being given orally to the yearling ewes

G ROBERTSTAD Was vaginal or preputial inoculation attempted with *Vibrio fetus* infected tissues?

V A MILLER No just with the *Vibrio fetus* culture

RUE JENSEN The question is pertinent since considerable experimentation has shown that it is difficult to produce abortions in pregnant ewes by feeding cultures of *Vibrio fetus* Abortions can be produced by feeding infected tissues

G ROBERTSTAD Since rams failed to transmit the disease by intrapreputial inoculation of a culture of *Vibrio fetus* perhaps intrapreputial inoculation with infected tissues should be tried

RUE JENSEN The next question is directed first to Mr Trueblood and then to Dr Miller

H T GIER What do you consider to be the normal mode of infection of *Vibrio fetus* in ewes?

MALCOLM TRUEBLOOD From case histories in the field and from experimental work, you can only assume that it must be a sanitation problem and therefore an ingestion of the organisms

RUE JENSEN Dr Miller do you have anything to add to that?

V A MILLER Only what we have seen with our own animals here in some of the pens when animals were aborting some of the ewes ate the infected membranes and this is considered as one of the normal modes of infection in sheep

RUE JENSEN This question is close to a very vital issue in understanding vibriosis of sheep It has been demonstrated conclusively that the disease can be transmitted by oral inoculation while experimentation conducted to date has not demonstrated a carrier condition in ewes following abortion nor transmissibility by coition If those data are correct where is the source of inoculation to the first aborting ewe? Once that first abortion occurs the disease can be transmitted by contamination of feed and water Since this is a vital question to understanding vibriosis of sheep I would appreciate anyone speaking to the subject from the audience Does anyone have suggestions?

MALCOLM TRUEBLOOD The Game and Fish Department of Wyoming had a problem with a herd of antelope in a certain area Noting a 13–15 / reproduction rate as compared to 150 or so in the normal areas and after requesting to check for vibriosis

we found a *Vibrio* like organism in 3 out of 25 antelopes This particular organism is very hard to culture and we could not send it to have it typed to determine whether it was a true *Vibrio fetus* or whether it was related to bovine *Vibrio fetus* or to ovine *Vibrio fetus* I personally feel it was the organism which we call *Vibrio fetus* This year they observed the reproduction rate again and have found several dead fawn but only one live fawn in this whole particular area indicating that the problem of losing the fawn is real Of the 25 we killed 24 were pregnant indicating that it is not a sterility problem Our station is working on a wild game reservoir, particularly in the antelope

RUE JENSEN Thank you Mr Trueblood

LLOYD C MOSS Does vibriosis occur in deer?

MALCOLM TRUEBLOOD I have not had an opportunity to work with deer The Game and Fish Commission in Wyoming has done some brucella studies using the blood of deer but as yet no vibriosis studies have been done

W G HUBER In the control animals on your oral inoculation did they receive the 50 cm³ of a so-called sterile tissue and if they did is there any possibility of causing abortion by an antibody reaction? Did the controls get the tissue too without the *Vibrio fetus* organism?

V A MILLER Control ewes were not given sterilized tissues

RUE JENSEN Next we have questions for Dr McEntee on the subject of vibriosis of cattle

ERVIN SMITH What effect has freezing of semen with or without antibiotic on the vibrio organism?

KENNETH MCENTEE Freezing of semen undoubtedly reduces the number of *Vibrio* organisms But they can be recovered whether or not antibiotics are added The problem concerns the safety of using frozen semen from *Vibrio* carrier bulls At present we do not have the answer We are working on this problem and hope eventually to come up with some information

G ROBERTSTAD Have pure cultures of catalase and hydrogen sulfide negative *Vibrio fetus* organisms been shown to cause abortions experimentally in cattle?

KENNETH MCENTEE That is a difficult question to answer dogmatically without trying to recall all of the published material on *Vibrio* transmission experiments Most of these reports appeared before the catalase and hydrogen sulfide tests were used to determine the characteristics of the *Vibrio* used There are numerous reports of abortion following the introduction of *Vibrio* organisms into the genital tract and by intravenous inoculations but I do not recall whether or not any of the reports indicated if the catalase activity and hydrogen sulfide activity were determined on the cultures prior to inoculation

H L EASTERBROOKS All I can add would be that some cultures that were used in early studies to produce experimental abortion would now satisfy the criteria used for pathogenic vibrios even though they have been kept for a long time since then on artificial media Whether or not they were catalase negative at the time of experimentation could not be proved now Because of reports of the isolation of catalase positive hydrogen sulfide positive vibrios from aborted fetuses I think that future experimenters will test the organism before and after experiments

RUE JENSEN Thank you Dr Easterbrooks

WAYNE SLETTEN A herd of 150 Hereford cows and 4 bulls experienced an outbreak of vibriosis resulting in ten abortions in December 1956 and January 1957

Now if no new female stock including heifers were introduced into this group for two breeding seasons and if the same bulls were used would the herd be apt to have self-eliminated the disease?

KENNETH MCENTEE I would say no because bulls appear to carry the *Vibrio* organism indefinitely Consequently the cows are going to be exposed continually Furthermore it has been shown that certain cows harbor the organism for a long period of time It would not be safe to assume that the herd would be free of the organism

MALCOLM TRUEBLOOD They report finding the catalase positive pathogen in aborted fetuses Dr McEntee is it true that you find the pathogenic catalase positive organisms in the genitalia of the bull and the sheath?

KENNETH MCENTEE Yes

RUE JENSEN Before leaving the subject of vibriosis I wish to say that in my opinion at least, it is of paramount importance to determine the cross-infectivity of vibriosis between sheep and cattle If cross infection is possible one species may act as a reservoir for the other If not, a different problem is presented

KENNETH MCENTEE Has a virus been isolated from vesicular venereal disease of cattle? If so is it related to the California virus of catarrhal vaginitis of cattle?

D G MCKERCHER To my knowledge a virus has not been isolated from the vesicular type of vaginitis but study indicates that there is no relationship between the virus vaginitis which we have reported on here this morning and the vesicular and granular types of vaginitis

KENNETH MCENTEE I was not referring to the so-called granular vaginitis but the condition in cattle characterized by vesicles and swelling of the vulva

H J HILL Would you please review the symptoms of the California virus infection in cattle?

D G MCKERCHER There is actually little I can add to what has been said this morning. The first indication of infection is the presence of small amounts of pus on the external genitals In many cases the pus will be observed to be adherent to the tail and to the buttocks Usually there will be a history of reproductive problems in such herds On examination of the vagina, one observes intense inflammation of the mucosa and in many cases inflammation and edema of the cervix On the floor of the vagina one will find considerable amounts of pus sometimes as much as 100 cm This will probably answer the question asked by Dr McEntee in that we did not observe any vesicular lesions on any part of the genital tract In the bull we have on one occasion only observed any indication of clinical infection This was a mild vesculitis which might or might not have been related to the infection However this bull was incriminated as being capable of transmitting the disease

H J HILL What interval is required for the disease to limit itself by immunity?

D G MCKERCHER This is a rather difficult one to answer I should emphasize again that we actually know very little about this vaginitis syndrome This is particularly true with reference to immunity I think I mentioned that we have made a limited serological survey and found that a high percentage of cattle in California carry antibodies to the vaginitis virus What the relationships of these antibody titers is to resistance and to past infection we do not know We have observed however that in a considerable number of cases we have been unable to infect our experimental animals which might suggest that the disease is more widespread than generally believed As far as the elimination of the disease by immunity development

is concerned I can only quote two examples From each of the two herds that I shall refer to a virus was isolated In the first herd only a few of the heifers had the infection They were affected for a relatively short time and then cleared up To our knowledge there has been no subsequent vaginitis infection in that particular herd The second herd was also one from which we isolated a virus In this herd the problem has been continuing now for about 3 years The infection flared up and many of the cattle became infertile—presumably from the infection Of course these animals are sold when they cannot be got with calf The disease will subside for a short time new animals are brought in and then it will recur I should think that over the last 3 year period from 40 to 50% of the cows in this particular herd have suffered from vaginitis infections It would appear therefore that the immunity plays little or no role in eliminating the infection from a herd Controlled experiments however might refute this

F X GASSNER First have you any therapy to combat this disease and second have you thought of influencing the response of the mucosa by the use of estrogenic agents or progestogenic agents? I refer to the reports in the literature and to some of our own that the estrogen stimulated mucosa is perhaps more susceptible to invading infective agents or aid in repair On the other hand hormonal treatment can prevent invasion It depends upon the relationship between the amount of estrogen to progestogen being available to the mucosa The infective state would differ in regard to the amount of estrogen progestogen or the ratio to each other present at that time which of course differs widely over the normal estrous cycle Have you or anyone else observed or done anything in that respect?

D G MCKERCHER In answer to your first question regarding therapy we have done practically nothing along this line On several occasions we injected streptomycin and penicillin but we are not able to say anything regarding the effectivenes of this therapy Miller in England in describing the disease which occurs in cattle there claims that they can clear up the infection in bulls by the parenteral injection of penicillin and streptomycin In infected cows they use acroflavin and glycerol as a douche which apparently does aid in clearing up the infection They have tried penicillin and streptomycin in infected cows but without feeling that it is of any particular value We have not tried treating bulls because of the absence of clinical infection in them

Insofar as the effect of estrogens in increasing the susceptibility of the vaginal mucosa is concerned we have thought about this possibility but we have not made a study of it The South African workers feel that cattle are probably more susceptible during estrus than they would be at other times but to my knowledge they have not investigated this particular possibility I think it is definitely one that should be looked into however

J C OSBORNE Would you please elaborate on this morning's statement that semen is infective yet the virus has not been isolated from semen?

D G MCKERCHER The evidence that the semen is infective is rather circumstantial inasmuch as certain bulls have been noted to transmit the infection consistently We have taken semen from such animals and succeeded in infecting heifers I cannot account for our having failed to isolate the virus from the semen of such bulls however I might point out in this connection that we made many attempts to isolate the virus from vaginal discharges from cattle While it so happened that our first attempt was successful we made at least fifteen or more attempts subsequently from discharges of cattle all of which manifested the typical clinical infection, before we isolated a second strain of virus Millar (1955 Viral infertility in cattle *Brit Vet J*

3 309) on the other hand readily isolates the virus from semen as well as from the discharge of infected cows Although circumstantial I feel there is good evidence that in the case of the disease which occurs in this country the agent is present in the semen although it does not produce any adverse effects on the semen in infected bulls that we have studied

A M SORENSEN Jr Dr McGowan, was there any observation made of injury in animals that had epididymitis?

BLAINE McGOWAN Yes we attempted to find some evidence or consistent pattern of injury attending this disease but there is none that we can discover That same story is told by the New Zealanders and the Australians We have seen this in ram lambs for example that have never been subjected to shearing. We have never found any scarsing or any other evidence of injury in and around the scrotum which would indicate that that was an avenue of infection Experimental inoculation indicates that no matter how or by what route the epididymitis organism is given it finally locates in the epididymis That makes us believe that this is a very tissue-specific organism

A M SORENSEN Jr Was there any therapy practiced on these animals?

BLAINE McGOWAN Aureomycin combined with Terramycin in massive doses has been used with limited success in some individual stud rams but the amount needed and the duration of treatment necessary to effect improvement would probably preclude its use on a practical basis in the usual commercial ram It may have some application in the individual stud ram Furthermore we are collecting a few rams now that were unilaterally infected and upon which we have performed unilateral castrations In fact, we have gone both ways We have taken the diseased testicle out of some of our rams and a normal testicle out of the other and we are starting to evaluate the semen from these rams Unilateral castration may prove of some value as a surgical means of therapy

CECIL BRANTON Dr McGowan was there an increase in morphologically abnormal sperm in these rams infected with epididymitis?

BLAINE McGOWAN Yes there was quite an increase in the abnormal forms Probably the most predominant abnormality was the separation of the head and the tail We also found many curled tails and quite a few protoplasmic droplets We found the epididymitis organism included in epithelial cells of which there was a high percentage Hence we had quite an array of morphological abnormalities and evidence of infection by epithelial cells

RALPH GANGAROSA Dr McEntee in an attempt to control vibriosis in cattle we could start with the Hereford herd you have mentioned earlier Would it be practical to run that herd on an infected basis that is would you get a suitable calf crop and is it practical to try to clear up this disease on a ranch basis once it appears If some attempt is made in controlling the disease and getting a calf crop by artificial insemination approximately how long would that procedure have to be kept up before you could start on a natural basis?

KENNETH McENTEE I think the most practical way of handling large numbers of beef cattle would be to operate on the basis of two separate herds if it can be done If they want to keep the old herd just let them run along with that and then establish a disease-free herd starting with virgin heifers and virgin bulls Artificial insemination is used with beef cattle but I realize that there are some problems If that can be done it will work very well

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II OVARIAN PHYSIOLOGY

R M COCKING Dr McEntee is it ever possible to determine with any degree o certainty that a herd is free from vibriosis after it has once been infected?

KENNETH MCENTEE The persistence of the organism in the bull is indefinite so t he remains the herd is always exposed to infection If the bull is eliminated most cows clear up in a few months However, there are a few reports of females remaining infected for approximately 400 days

R M COCKING What if artificial insemination is used in the herd?

KENNETH MCENTEE Artificial insemination should eliminate it It will eliminate the majority of the trouble in a relatively short time

LLOYD C FAULKNER Dr McEntee are there any controlled experiments on the parenteral use of antibiotics in vibriosis in cattle?

KENNETH MCENTEE Adler has reported on that and it can be concluded from his experiment that such therapy is ineffective

RALPH GANGAROSA Dr McEntee if vibriosis is found in a purebred herd and some bulls are to be sold out of that herd how many tests would you expect it would take to say that there is assurance that the bulls are suitable to sell?

KENNETH MCENTEE I would not care to make a definite statement on that Dr Hughes cultured one known infected bull fourteen times before he re isolated the organism

J C OSBORNE Dr McEntee should I interpret your answer as meaning that the bull cannot be treated for vibriosis successfully?

KENNETH MCENTEE No I think successful treatments have been used I was referring mostly to breeding studs with large numbers of bulls I think the main problem is that of reinfection We have to eliminate that possibility I don t think it will be too difficult to perfect a treatment procedure then

R B LANK Dr McEntee has it ever been shown that a catalase negative strain would change to a catalase positive strain of vibriosis in your studies?

KENNETH MCENTEE Not that I know of

RUE JENSEN Mr Trueblood would you like to comment on that

MALCOLM TRUEBLOOD I have never seen one change from a catalase positive into a catalase negative but I have seen them change from pathogenic to non pathogenic.

RALPH GANGAROSA Dr McEntee I don t feel that one question I asked was quite answered If you had a herd with vibriosis and you were artificially inseminating, do you feel that after a period of time say one year or two years on that program then using the same cows and starting out with a clean bull could you revert to the natural way?

KENNETH MCENTEE I think you eventually can but the question concerns when will it be safe I would not dare to make a definite statement in that regard It is probable that most of the infection would have been eliminated within a year or two I hesitate to pin it down I don t think a person can do that with the evidence avail able now

RECENT STUDIES ON THE MECHANISM OF OVULATION IN THE COW*

WILLIAM HANSEL D T ARMSTRONG and KENNETH MCENTEE
Animal Husbandry Department N Y State College of Agriculture and Department of Pathology and Bacteriology N Y State Veterinary College Cornell University

EXPERIMENTS carried out in recent years have provided evidence that the release of pituitary gonadotropin(s) necessary for ovulation in the cow as well as in other spontaneously ovulating species is controlled through the hypothalamus This evidence has been reviewed by Hansel (1957) Atropine administered at the beginning of estrus was found to block ovulation in a large percentage of cases in the cow Ovulation occurred at the same time or somewhat earlier when both atropine and chorionic gonadotropin were administered at the beginning of estrus These findings indicate that the bovine anterior pituitary gland is stimulated to release the luteinizing or ovulatory hormone during estrus by a neurogenic mechanism having a cholinergic component Progesterone administered at the beginning of estrus was found to hasten ovulation in the cow while estradiol administered at the beginning of estrus did not hasten ovulation Ovulation remained blocked in a similar proportion of heifers when either atropine alone or atropine plus progesterone were given at the beginning of estrus The latter result suggests that the ovulation hastening effect of progesterone administered at the beginning of estrus is mediated through the hypothalamus rather than by direct action on the anterior pituitary Relatively small amounts of epinephrine administered at the beginning of estrus did not hasten ovulation in cows

These results and the results of numerous experiments conducted with laboratory animals and summarized by Markee Everett and Sawyer (1952) and Harris (1955) have given rise to a concept of neurohumoral control of the release of the pituitary gonadotropic hormone(s) necessary for ovulation The basic features of this concept include (1) one or more neurohumoral substances released by certain nuclei in the hypothalamus as a result of the stimulus of copulation in induced ovulating species and unknown stimuli in spontaneously ovulating species (2) the transport of this chemical media

* Supported in part by funds from the regional project NE 1 entitled Causes and Prevention of Reproductive Failures in Dairy Cattle a co-operative study by agricultural experiment stations in the north eastern region and the Dairy Husbandry Research Branch U S D A

The possibility that certain neurohypophyseal hormones or related sub stances may be concerned with the activation of the anterior pituitary has recently been postulated as a result of the discovery that these hormones (oxytocin and vasopressin) appear to be formed in hypothalamic nuclei rather than in the posterior lobe of the pituitary itself (Bargmann and Scharrer 1951 Scharrer and Scharrer 1954) Neuro-secretory material which stains selec tively with chrome hematoxylin (Gomori 1941) has been described as originat ing in the supraoptic and paraventricular nuclei and migrating down the nerve axons to their endings in the posterior pituitary Upon passage through the median eminence these axons come in very close approximation to the primary capillary plexus of the hypophyseal portal blood vessels As a result of these anatomical relationships it has been proposed by Benoit and Assen macher (1953) and Scharrer and Scharrer (1954) that the chemotransmitter substance responsible for controlling the gonadotrophic functions of the pituitary may be contained in this Gomori staining material There is now considerable evidence (reviewed by Zuckerman 1954) that the material being stained is a carrier substance for the posterior lobe hormones rather than the hormones themselves Recent studies by Adams and Sloper (1956) of pitui tary and hypothalamic sections stained by a histochemical procedure specific for cystine have shown cystine to have practically identical distribution with that of the neuro-secretory material described by Bargmann and Scharrer (1951) They conclude that since the neurohypophyseal hormones contain cystine they are staining the hormones themselves by this technique and they feel that it provides good confirmation of the hypothalamic origin of these hormones

Thus if one or more of the neurohypophyseal hormones is actually respon sible for anterior pituitary activation a close integration of posterior and anterior lobe function is implied As far as gonadotropic hormone release is concerned there is considerable indirect evidence to support such an integra tion It is well known that mating in the rabbit provides the neural stimulus for the release of the ovulatory hormone from the anterior pituitary and there is evidence that a concomitant release of oxytocin also occurs as Reynolds (1930) has recorded increased uterine motility following mating In fact in the rabbit an increase in uterine contractions has been observed to occur following massage of the vulva (Krehbiel and Carstens 1939) and this may be correlated with the observations of Hammond (1925) that this sort of a stimulus in the presence of the male will sometimes cause ovulation in the absence of coitus

Similar supportive evidence for an integration of the posterior lobe hor mones and gonadotropin release is available for the cow since Vandemark and Hays (1952) have shown that sexual stimulation including sight of the bull sniffing of the vulva mounting and actual copulation all cause increased uterine motility The same workers (1953) demonstrated that manual manipu lation of the bovine reproductive tract caused an output of oxytocin as

tor(s) to the blood sinusoids of the anterior pituitary, probably by the hypophyseal portal vascular system where it causes the release of the gonadotropin necessary to cause ovulation, (3) this neurohumoral release mechanism appears to contain both a cholinergic and an adrenergic component It has also been suggested that epinephrine is the final humoral agent that activates the anterior pituitary

Jubb and McEntee (1955) described a process of rapid degranulation occurring in the small basophils (delta cells) of the bovine anterior pituitary during the early hours of estrus This process begins in the medulla adjacent to the zona tuberalis and sweeps on a broad front through the whole pars distalis proper Degranulation of the delta cells was not seen in cows which came in estrus and failed to ovulate The striking affinity of the delta cells for the blood sinusoids in the anterior pituitary and their degranulation at the approximate time when atropine administration has been found to block ovulation strengthened the concept of gonadotropin release in response to a neurohumor carried in the blood from the hypothalamus to the anterior pituitary In addition the delta cell degranulation process has provided a useful new experimental technique applicable to ovulation studies in the cow Obviously the next question to be answered is whether or not atropine blockage of ovulation also blocks pituitary delta cell degranulation and some of the studies described in this report were designed to answer this question

As knowledge in the area of pituitary–hypothalamic relationships has accumulated it has become clear that two additional questions must be answered before this knowledge can be applied in a practical way to the problem of increasing reproductive efficiency in our farm animals These questions are (1) What exteroceptive pathways to the hypothalamus are involved and what stimuli most effectively activate them in various species? (2) What is the chemical nature of the neurohumor(s) acting between the hypothalamus and anterior pituitary? The latter question will be considered in some detail in this report

The conclusion of Sawyer Markee and Everett (1950) that epinephrine is the final humoral agent which activates the anterior pituitary is open to question Large dosages of epinephrine are necessary for induction of ovulation when administered intravenously to atropinized rabbits Unphysiological substances such as copper acetate have also been shown to induce ovulation when injected either intravenously (Fevold Hisaw and Greep 1936) or directly into the pituitary (Sawyer and Markee 1950) Relatively small doses of epinephrine given at the beginning of estrus did not hasten ovulation in cows (Hough Bearden and Hansel 1955) Donovan and Harris (1956) reported that epinephrine injected directly into the pituitary gland did not cause ovulation in rabbits when the pH of the solution was adjusted to 6 9–7 1 Three of fifteen rabbits ovulated when an acid epinephrine solution was injected indicating that the pH of the epinephrine solution injected influenced gonadotropin release

humoral mediator which brings about gonadotropin secretion following appropriate stimuli that acetylcholine from nerve endings in the anterior hypothalamus is responsible for the oxytocin release and that it is the acetyl choline phase which is blocked by chlorpromazine

While these experiments on the surface appear to offer a good explanation for the hypothalamic control of gonadotropin secretion there may be some doubt as to whether the urinary 17 ketosteroid fractions which they have observed to increase are actually of testicular origin Nishikawa *et al* (1955) have demonstrated high titers of the same fractions in the urine of females and of castrate males suggesting that they are probably of adrenal rather than gonadal origin Further pitocin was used in the experiments rather than purified or synthetic oxytocin and although this preparation was found to be free of gonadotropin the possibility that it contained other impurities including vasopressin cannot be ignored A considerable number of recent reports show that vasopressin or something closely associated with it causes pituitary ACTH release which results in adrenal cortical secretion (McCann and Brobeck 1954 Guillemin and Hearn 1955 McDonald Weise and Patrick 1956 Swingle *et al* 1956) Shibusawa *et al* (1955) may really be describing an adrenal cortical rather than a gonadal response in their experiments and their evidence requires further confirmation

Benson and Folley (1956) have reported that oxytocin injections in lactating rats markedly retard the mammary gland involution that normally occurs following removal of the suckling young These results indicate that the release of prolactin and perhaps other anterior pituitary hormones concerned in lactation can be stimulated by treatment with oxytocin

Because of these indirect indications that oxytocin of hypothalamic origin might be involved in regulating anterior pituitary gonadotropin secretion it was decided to test the effect of injected oxytocin on ovulation time in dairy heifers

EXPERIMENTAL PROCEDURES

In order to obtain information on whether atropine blockage of ovulation also blocks pituitary delta cell degranulation in the bovine seven heifers were treated with atropine at the beginning of estrus and slaughtered at intervals ranging from 14 to 47 hours thereafter The atropine was injected subcutaneously at the rate of 40 mg/kg of body weight a dosage known to block ovulation in about 70% of the heifers to which it is administered The heifers were stunned by a blow on the head bled and the pituitary removed as quickly as possible The whole pituitary gland was split in a mid sagittal plane and one half of the gland was fixed in Bouin s solution and the other half in 4% aqueous formaldehyde The tissues were dehydrated in alcohols imbedded in paraffin sectioned at 4μ and stained by the periodic acid Schiff technique of Pearse (1952)

measured by increased intra mammary pressure These observations can be correlated with those of Marion *et al* (1950) that sterile copulation in the cow caused ovulation to occur significantly earlier than in unmated controls, suggesting an earlier release of gonadotropin as a result of the increased secretion of oxytocin

While these experiments suggest a close relationship between oxytocin re lease and secretion of gonadotropins none of them offer direct proof that the latter are secreted as a result of the former It is quite possible that some other substance which is released concomitantly with oxytocin is the factor which stimulates anterior pituitary gonadotropin release There is evidence that the other known neurohypophyseal hormone vasopressin is often re leased simultaneously with oxytocin when teleology would call for the libera tion of only one of the hormones Thus the act of coitus, known to cause increased uterine contractions perhaps for the purpose of assisting in sperm transport and apparently due to oxytocin liberation has been shown to cause a decrease in water diuresis in the human (Friberg 1953) and rats (Eränkö, Friberg and Karvonen 1953) Presumably the latter effect is due to the con comitant liberation of the antidiuretic hormone (vasopressin) Anti diuresis has also been shown to occur in the lactating rabbit following suckling a stimulus known to cause oxytocin output (Cross, 1951) Conversely an in crease in osmotic pressure of the blood evoked by the intravenous injection of a concentrated NaCl solution a stimulus which causes release of the anti diuretic hormone (Verney 1947) has also been shown to result in milk ejection (Andersson 1951) and increased uterine activity (Abrahams and Pickford 1953) suggesting a simultaneous liberation of oxytocin

Thus the gonadotropin secretion which has been described as being asso ciated with stimuli which cause oxytocin release could quite conceivably be due to vasopressin or indeed to some other as yet unidentified substance of hypothalamic origin which is liberated at the same time

The belief that oxytocin actually stimulates gonadotropin secretion has been expressed in a recent series of papers by Shibusawa *et al* (1955a 1955b 1955c) These workers have demonstrated an increase in certain chromatographic fractions of urinary 17 ketosteroids of human males following the injection of pitocin and believe that these fractions represent androsterone and etio cholanolone These fractions are presumed by the authors to be of testicular origin and under the influence of gonadotropin secretion induced by the pitocin injections They also observed slightly increased testicular weights of injected rats but these may have been merely reflections of increased body weights which were also observed

These workers (1955b) later demonstrated an inhibition of gonadotropin release by the tranquilizing drug chlorpromazine and were able to override this inhibition either by the injection of pitocin or acetylocholine (1955c) From these observations they concluded that oxytocin is the natural neuro

ovulation would normally have occurred In these five cases atropine had blocked ovulation yet delta cell degranulation was nearly complete in two cases and had occurred to a considerable extent in two other cases

TABLE I

The Effect of Atropine Administered at the Beginning of Estrus on Pituitary Delta Cell Degranulation in Dairy Heifers*

Heifer No	Hours from beginning of estrus to slaughter	Ovarian status	Degree of degranulation of pituitary delta cells
103	47	Ovulation blocked	Partial
104	44	Ovulation blocked	None (fully granulated)
110	42	Ovulation blocked	Partial
94	42	Ovulation blocked	Nearly complete
112	33	Ovulation blocked	Nearly complete
117	14	Large follicle	Nearly complete
124	14	Large follicle	Partial

40 mg/kg body weight subcutaneously

Figure 1 illustrates the fully granulated delta cells found in the pituitary of a heifer slaughtered 44 hours after the beginning of estrus and atropine ad ministration Figure 2 illustrates the nearly complete degranulation seen in another heifer slaughtered 42 hours after the beginning of estrus and atropine *administration Ovulation was blocked in both cases These results empha* sized the need for more data on the delta cell degranulation process in normal animals at known times during this critical period and accordingly the data shown in Table II have been collected

TABLE II

Pituitary Delta Cell Granulation in Late Proestrus and Early Estrus in Dairy Heifers

Heifer no	Stage of cycle at slaughter	Size of largest ovarian follicle (cm)	Degree of degranulation of pituitary delta cells
61	19 days postestrus	1 1	None (fully granulated)
5	23 days postestrus	1 2	None (fully granulated)
	In Estrus		
126	20 min	1 3 × 1 7	Nearly complete
120	30 min	(Large)	Partial
107	1–2 hr	1 4 × 1 2	Nearly complete
130	2–3 hr	1 × 1 2	Nearly complete

During the conduct of this experiment it became obvious that too little was known in regard to variations in the time of delta cell degranulation in normal heifers Consequently 6 additional heifers were slaughtered at very accurately determined times during the critical hours just prior to and after the beginning of standing estrus The heifers slaughtered after estrus were checked with a teaser bull at 2 hour intervals until they appeared to be approaching estrus after which they were checked at approximately 30 minute intervals They were slaughtered as rapidly as possible after they came in standing estrus The pituitaries of these heifers were fixed sectioned and stained as described above

In the experiments to determine the effect of exogenous oxytocin on ovulation time in dairy heifers the length of estrus and the time of ovulation were determined in each heifer in a control and on oxytocin treated period The heifers were tested for estrus at 2 hour intervals by a teaser bull Ovulation time was determined by rectal palpations performed at 2 hour intervals In some cases the control period preceded and in other cases followed the treatment period The oxytocin was administered as soon as possible after the beginning of estrus Each animal received an intravenous injection of 50 or 60 units of Armour s Purified Oxytocin Principle* in addition to a subcutaneous injection of 50 or 100 units of the same preparation A subsequent experiment to determine the ability of oxytocin to overcome atropine blockage of ovulation was conducted in the same way except that 40 mg/kg of body weight of atropine was administered along with the oxytocin at the beginning of estrus To date 11 heifers have been used in the first experiment and 6 in the second experiment

In addition 63 immature hypophysectomized rats have been used in assays to detect the possible presence of gonadotropins in the oxytocin preparations used These rats were injected subcutaneously once daily with 2–5 Units doses of various oxytocin preparations for periods of 4–11 days after which they were sacrificed and the uteri and ovaries removed and weighed Appropriate groups of control hypophysectomized rats were injected with the solution in which the oxytocin was dissolved

RESULTS

The results of the experiment in which the degree of delta cell degranulation was studied in heifers given atropine at the beginning of estrus and slaughtered at various time intervals thereafter are summarized in Table I Although the delta cells in one heifer were still fully granulated when she was slaughtered 44 hours after the beginning of estrus and atropine administration these cells had undergone a considerable degree of degranulation in all of the other heifers The degranulation process was nearly complete in 3 heifers 1 of which was slaughtered 14 hours after the beginning of estrus Five heifers were slaughtered at 33 to 47 hours after the beginning of estrus at which time

* Kindly supplied by Dr Irby Bunding, the Armour Laboratories Chicago Ill.



FIG 1 Fully granulated delta cells in the pituitary of a heifer killed 44 hours after atropine treatment at the beginning of estrus Ovulation was blocked PAS ×310 Heifer 104

The degranulation process was nearly complete in 3 of 4 heifers slaughtered in the first 3 hours of estrus and the delta cells of the fourth heifer slaughtered 30 minutes after the beginning of standing estrus were partly degranulated Heifer No 126 in which degranulation was nearly complete 20 minutes after the beginning of standing estrus is of particular interest This heifer would mount other heifers and was quite excitable 6 hours before she would stand when mounted by a teaser bull Figure 3 illustrates the heavy accumulation of PAS—positive granules that normally occurs during proestrus Figure 4 shows an area of nearly complete degranulation and an area in which delta cell degranulation has not yet occurred in the pituitary of a heifer slaughtered 30 minutes after the beginning of standing estrus

TABLE III

Effect of Oxytocin Administered at the Onset of Estrus upon Estrus Length and Time of Ovulation in Dairy Heifers

No of heifers	Treatment	Duration of standing heat (hours)	Time from end of estrus to ovulation (hours)	Time from onset of estrus to ovulation (hours)
11	Untreated	20 3	11 8	32 1
11	Oxytocin treated*	21 2	6 8†	28 0

* 50–60 U S P units oxytocin intravenously plus 50–100 units oxytocin subcutaneously at onset of estrus

† Significantly different from untreated heifers ($P<0\ 01$)

TABLE IV

Effect of Atropine plus Oxytocin† Administered at the Beginning of Estrus on Ovulation Time in Dairy Heifers*

Heifer no	Hours from beginning of estrus to ovulation
106	240
120	144‡
107	150
113	156
122	30
121	Failed to ovulate for two weeks

* 40 mg atropine sulphate per kg body weight subcutaneously

† 50 U S P units of Armour s Purified Oxytocin Principle intravenously and 50 units of the same preparation subcutaneously at the onset of estrus

‡ Returned to estrus after approximately 126 hours.



FIG 3 The normal accumulation of granules in the delta cells of a heifer killed at the nineteenth day of the estrous cycle PAS ×440 Heifer 61



FIG 2 Nearly complete degranulation of the delta cells in the pituitary of a heifer killed 42 hours after atropine treatement at the beginning of estrus Ovulation was blocked PAS × 440 Heifer 94



FIG 4*b* (see legend at FIG 4*a*)

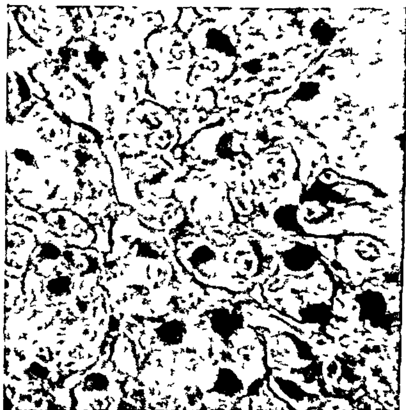


FIG 4*a* Degranulated and granulated delta cell areas from the pituitary of a normal heifer slaughtered after being in estrus for approximately 30 minutes The degranulation process had proceeded only part way down the core area of the pars distalis in this case PAS × 440 Heifer 120

TABLE V

Results of Gonadotropin Assay of Oxytocin Preparations using Hypophysectomized Rats

No of rats	Daily dose of oxytocin (U.S.P units)	Duration of injection period (days)	Mean uterine weight (mg)	Mean ovarian weight (mg)
8	none	4	14 1	7 7
7	2 (Armour)	4	12.9	7 9
7	5 (Armour)	4	13 4	8 5
12	none	11	14 6	6.5
6	4 (Armour)	11	14 4	6 6
10	none	11	13 3	6 0
13	2 (International Hormones Inc.)	11	14 5	5.5

The results of the experiment in which oxytocin was administered at the beginning of estrus are summarized in Table III The length of estrus was not significantly different in the control and treated periods but the time elapsing from the end of estrus to ovulation was significantly (P<0 01) decreased by the oxytocin treatment Actually the time from the end of estrus to ovulation was shortened in 7 of the 11 heifers and appeared not to be affected at all in the remaining four The total time elapsing from the beginning of estrus to ovulation was not significantly different in the treated and untreated periods

Although oxytocin shortened the length of time from the end of estrus to ovulation it was not effective in overcoming the blockage of ovulation by atropine as may be seen from Table IV Ovulation occurred at the normal time in only 1 of the 6 heifers treated Atropine alone does not block ovulation in every case

The results of the assays of the oxytocin preparations used in these studies as well as one other oxytocin preparation are shown in Table V No significant increase in either uterine or ovarian weight was noted after the injection of any of these preparations in amounts up to 5 U S P units per day for periods as long as 11 days

DISCUSSION

The results of the studies of pituitary delta cell degranulation show that atropine is capable of blocking ovulation even when the degranulation process has occurred Actually the process seems to occur slightly earlier in normal animals than Jubb and McEntee (1955) previously reported and had probably occurred to a considerable extent before the atropine was administered in most cases The one case in which the delta cell degranulation did not

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occur at all in an atropinized heifer, slaughtered after 44 hours and th cases in which only partial degranulation had occurred suggest that atrop may have blocked the degranulation process in those cases where it had occurred or was not complete by the time the atropine was administer These results suggest that atropine blocks ovulation in the cow by block some pituitary process that normally occurs later than the delta cell degra lation and is presumably related to LH release The delta cell degranulat may well represent the FSH release which causes pre ovulatory swelling of follicle and the manner in which the process occurs suggests that it too may under the control of some humoral substance in the blood sinusoids

After it was found that oxytocin shortened the interval between the end estrus and ovulation it became necessary to determine if the preparation u contained gonadotropins The assays conducted with hypophysectomized r showed that gonadotropins were not present in measurable amounts in oxytocin preparation used It is not known whether the results obtained due to oxytocin *per se* or to vasopressin or some other substance contai in the oxytocin preparation used The dosages of oxytocin used failed to ov come atropine blockage of ovulation in fact chorionic gonadotropin is only substance tested to date which has been effective in causing ovulation atropinized heifers If atropine also blocks some pituitary change occurr later than the delta cell degranulation as the data suggest it is quite conce able that oxytocin might hasten ovulation in normal animals and yet be effective in overcoming the atropine blockage of one of the two processes will be interesting to study the effects of various oxytocin preparations on delta cell degranulation process and also to test the effects of prolactin ovulation time in view of the report of Benson and Folley (1956) that oxyto injections prolong prolactin secretion in the lactating rat

SUMMARY AND CONCLUSIONS

Evidence for the hypothalamic control of pituitary gonadotropin secreti in the cow and other species has been reviewed

The degranulation of periodic acid Schiff staining material in the sm basophils (delta cells) of the bovine pituitary has been studied in normal heif and heifers treated with atropine at the beginning of estrus Delta cell d granulation occurs slightly earlier in normal animals than was previous reported Atropine is capable of blocking ovulation even when the degranul tion process has occurred Oxytocin administered at the beginning of estr decreased the time interval between the end of estrus and ovulation This effe was not due to gonadotropins contained in the oxytocin preparation use Oxytocin in the dosages used did not overcome atropine blockage ovulation in the cow Oxytocin or some substance other than gonadotropi contained in the preparations used affects ovulation time in dairy heifers

A CYTOLOGICAL STUDY OF THE MATURATION PROCESS OF THE OVUM OF THE EWE DURING NORMAL AND INDUCED OVULATION

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IN THE reproductive process of monotocus animals the egg assumes a major role in comparison with the male germ cells At least 20% of the sperm cells in a semen sample may be abnormal and such a sample can still produce a high percentage of conceptions However when the egg is abnormal in any of its various capacities subsequent estrus cycles are necessary to obtain fertiliza tion The egg undergoes rather critical cellular changes during the process of maturation When these changes do not occur on schedule the result is an egg that is incapable of being fertilized

Since ewes that have been induced to ovulate by using gonadotropic hor mones have had low conception rates a cytological study was made of the maturation process of the eggs from such ewes in order to determine if this low fertility could be attributed to the production of immature eggs The pro cesses of maturation as exhibited by the normal and induced ovulated ewes was studied on a comparative basis

As early as 1875 Van Beneden s work on the maturation divisions in the rabbit ovum initiated an interest for investigations in other mammals Since this work polar body formation has been described for the mouse by Sobotta (1895) Kirkham (1906) Long (1911) Allen (1923) and Snell (1940) for the guinea pig by Moore (1908) for the bat by Van der Stricht (1923) for the rat by Sobotta and Burckhard (1911) Kirkham and Burr (1913) and Blandau (1945) for the cat by Longley (1911) and Dawson (1940) for the monkey by Corner (1923) Allen (1930) *and Hartman and Corner (1941) for the human* by Thompson (1919) Hoadley and Simons (1928) Rock and Hertig (1944) Hamilton (1944) and Shettles (1953) for the cow by Hartman *et al* (1931) and for the sow by Corner (1917) and Spalding (1955)

The above investigators found that in general the maturation pattern exhi bited by the eggs in these mammals follow a uniform scheme The egg nucleus undergoes one division and initiates a partial second division just prior to ovulation Thus at the time of ovulation the normal egg possesses one polar body and the nucleuse is in metaphase II of the second division

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egg nucleus remains quiescent and vesicular The nucleus lies toward one side of the egg and contains several conspicuous nucleoli along with the chromatin granules (FIG 1*c*) After the ewe is in estrus for several hours the nucleus goes into the meiotic prophase stage This is characterized by the presence of one or more darkly stained nucleoli and long paired chromosomes Subsequently the nucleoli and the nuclear membrane disappear and the chromosomes become short and compact (FIG 1*d*) The chromosomes are then oriented into a metaphase plate which is located near the periphery of the ovum (FIG 1*e*) This first metaphase is of short duration and the chromosomes quickly divide and migrate to opposite poles (FIG 1*f*) The nucleus is *now prepared for the formation of the first polar body It appears that the* first polar body is abstricted by a furrowing process in the cytoplasm surrounding the peripheral nucleus Upon completion of abstriction the polar body is completely surrounded by a membrane and becomes a separate and independent structure (Fig 1*g*) The egg nucleus immediately forms the second metaphase plate It is in this stage i e one polar body formed and the chromosomes in metaphase of the second division that the egg leaves the follicle at ovulation (Table I)

If fertilization is successful the nucleus completes the second maturation division and a second polar body is abstricted (FIG 2*a*) In the fertilization process many of the eggs showed that the head neck and middle piece of the

TABLE I

Cytological Data of Follicular Ova Recovered Prior to Ovulation from Ewes in Estrus

Ewe No	Hours in estrus	Number of ova examined	Maturation stage of nucleus
39A	19	2	Vesicular metaphase I
3A	7	1	Prophase
32	7	3	Prophase
72	7	1	Metaphase I
70	19	1	Prophase
51	27	1	Metaphase II
8A	16	2	Vesicular metaphase I
20A	16	1	Metaphase I
24A	16	1	Prophase
66	16	1	Metaphase I
23A	16	1	Metaphase II
31A	16	2	Prophase metaphase I
71	24	1	Metaphase II
30A	18	1	Metaphase II
41A	18	1	Metaphase II
43	18	1	Metaphase I
6B	24	5	Metaphase II (4) vesicular

Approximate number of hours

Variation from the above pattern was reported by Evans and Cole (1931) for the dog by Pearsons and Enders (1943) for the fox and by Hamilton (1945) for the horse In these three groups of animals they found that the first polar body was not formed until the ovum reached the oviduct

Cytological studies of the maturation of eggs induced to ovulate by hor mone treatments are meager Mordicard (1940) obtained polar body forma tion in adult virgin rabbits by the injection of chorionic gonadotropins directly in the ovarian follicle Eggs from sows that had been induced to ovulate by injections of gonadotropins were observed by Spalding (1955) to follow the same maturation process as normally ovulated eggs

METHODS AND MATERIALS

More than 800 ova were obtained from follicles from the oviducts and from uteri of slaughtered ewes Follicular ova were obtained by aspirating the fluid from the follicles with a syringe fitted with a 22 gauge hypodermic needle Tubal and uterine ova were obtained by irrigating this portion of the tract with isotonic saline solution The recovered ova were fixed in acetic alcohol (1 3) and stained as whole mounts in aceto-carmine The follicular ova were dissected from the granulosa cells before being mounted for study

One hundred and forty nine grade rambouillet ewes were used in this study Twenty five of these animals were classified as being in anestrum and 124 were cycling

The hormone used to produce ovulation was furnished by Armour Research Laboratory of Chicago Illinois It is predominately the follicle stimulating fraction (FSH) of the pituitary gonadotropic hormone Since this hormone did not produce estrus stilbestrol was injected simultaneously with the gonado tropic hormone when estrus was desired for mating

OBSERVATIONS

Maturation of the Normal Ovum

The maturation process may be considered from two areas One is con cerned with extra ovum changes that occur in the granulosa cells which sur round the egg and the other chromatic changes in the nucleus

Before the animal comes into estrus the egg is tightly encased within the cumulus oophorus (FIG 1*a*) During middle or late estrus these cells are somewhat dispersed and the egg while still within the follicle there becomes surrounded by a gelatinous mass interspersed with scattered granulosa cells This viscous plug surrounds the egg until after ovulation (FIG 1*b*) but shortly after the egg enters the fimbriated end of the oviduct this mass of loosely adhering cells along with the cells of the zona radiata is dispersed from the zona pellucida of the ovum This change in the consistency of the fluid immediately surrounding the egg was always found to be associated with nuclear maturation

During the development of the primary secondary and tertiary follicle the





F[illegible]

[illegible] 400

b A [illegible] the [illegible] 350

Whole [illegible] 47

d N[illegible] 838

N[illegible] the first [illegible] 400

f N[illegible] form [illegible] 800

g [illegible]

sperm penetrated deep in the cytoplasm before coming to rest The sperm head and the ovum nucleus are transformed into male and female pronuclei (FIG 2b) The pronuclei fuse and form one nucleus The chromosomes in this nucleus soon regain their identity and align themselves in the first mitotic metaphase (FIG 2c) The nucleus then divides by mitosis forming the first segmentation spindle Shortly thereafter cytokinesis occurs and two-celled embryo is formed (FIG 2d)

If fertilization does not occur the achromatic spindle of the second meiotic division of the ovum undergoes a series of changes resulting in complete degeneration While the non fertile eggs are still in the region of the oviduct, the achromatic spindle of the second meiotic division breaks down and the chromosomes scatter at random into the cytoplasm (FIG 2e, f and g) Subsequently the scattered chromosomes become small pycnotic masses The vitellus eventually fills the entire zona pellucida and the cytoplasm begins to degenerate Sometimes the ovum undergoes fragmentation which may or may not be accompanied by some nuclear divisions

The spindle seems to be the shortest lived structure in the mature egg The first visible degenerative changes are manifest by a breakdown of the spindle fibers and scattering of the chromosomes into the cytoplasm Later cytoplasmic extrusions are evident at the periphery of the egg Subsequently the cytoplasm appears shrunken within the zona and the chromatic material is pycnotic or completely disintegrated

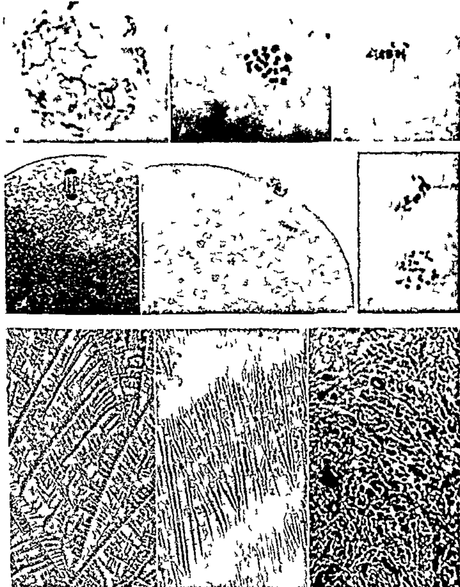
Maturation of Ova in Treated Ewes

The ova obtained from ewes that were treated with gonadotropins followed the same maturation pattern as that of normal maturing eggs The nucleus of the follicular eggs underwent the first meiotic division and reached metaphase II before ovulation occurred (Tables II and III) Cytologically no difference was detected in normally ovulated eggs and eggs from induced ovulations (FIG 3a–f)

Of the 14 tubal eggs obtained from the treated anestrous ewes 11 were in the metaphase II stage one showed a spindle breakdown one was fragmenting and one had cleaved into two blastomeres The three uterine ova from these animals yielded one that was pycnotic and two in metaphase II (Table II)

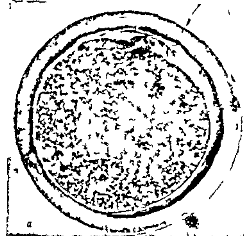
Data from the ova recovered from treated luteal phase ewes slaughtered 40 –63 hours after injections of FSH yielded 41 tubal ova in metaphase II and 3 that had a pycnotic nuclei or spindle breakdown Ova recovered from the uteri of these same animals showed 19 in metaphase II and 21 with pycnotic nuclei (Table III) Some of the eggs with pycnotic nuclei may have been from the previous ovulation

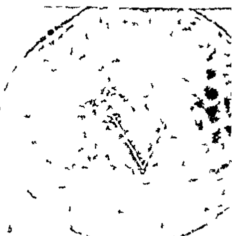
It is of interest to note that in many of the follicles that were not adequately stimulated to produce ovulation the nuclear maturation was initiated in slightly more than one half of the eggs obtained from such follicles that were 3 mm and over in diameter (Table IV) The ultimate fate of such stimulated



F G 3

Wh l m t faf ll l m o df m t t d w ho g l m t po-
phas t g F ll l d m te 4 mm 800
b Wh l m t ff ll l mf m t t d w h w g p l w f th l t m t m t
ph se fig 800
Wh l m t ff ll l o mf ma t t d w h w g l t al w f th l t m t m t
ph fig 800
d Wh l m t ff ll l mf m t ted h w g th l t m ot t l ph fig w th
lyf m t f th m d b dy 400
Wh l m t f m t l t b l m d f om t ted h w g th l t p l b dy
mpl t ly t ded d l t l w f th d m t m t ph fig 400
f A t b l o mf m t eat d h g th fi t p l bod d p l w f th se d m t
ph II 800
g A fl m d d cerv l mear f m w m l t s h w g typ l f l k p tt 800
h A fl m d d l m f m w th t h d 24 h p ly bee g 35
A m U t fg d t p d 15 mg f t lb l Th p tt s l fi d atyp l 800
A fl m d d ce l m f m l t l h t h d 24 h p e ly b g 35 A m
U t fg d p d 15 mg f t lbe t l Th g t p tt 800











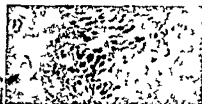




FIG 2

a H g dr pmo t f f rtl do m h w g the l t d 2 d pol bod e loc ted b t cen tl yt pl m of th eg d th o pell d 400
b Whole mo t f a f rtl d t b l o m how g m l d f m l p n cl d a p l r b dy (See r s) d t g l t n of th p l) 700
Chro o mes f om f rtl d t b l gg of P l w of th m tot c m t ph se f th fi t gme t t l a g (D pl d numbe of h mosom = 54) 800
d Two-c ll d mb y f o no d t of e h w g l t l w of th h m t c f g es 400
Whol mou t f g d t m ll t t g a ly p) of th hrom om d d g a to of the l t p l body 838
f N cl u f ma g dt b l o m how g p ndl b kd w d th dom sc tte g of ch mo- m s 800

TABLE II

Cytological Data from Ovulated Ova Recovered from Treated Anestrous Ewes

Tubal ova		Uterine ova	
No ova examined	Maturation stage	No ova examined	Maturation stage
11	Metaphase II	2	Metaphase II
1	Spindle breakdown	1	Pycnotic
1	Fragmenting		
1	Two-celled stage		

TABLE III

Cytological Data from Ovulated Ova Recovered from Treated Luteal Phase Ewes

Tubal ova		Uterine ova	
No ova examined	Maturation stage	No ova examined	Maturation stage
41	Metaphase II	20	Metaphase II
1	Spindle breakdown	21	Pycnotic
1	Pycnotic		
3	Fragmenting		
2	Diploid		

TABLE IV

Cytological Data of Follicular Ova from Treated Ewes

	Proestrum	Anestrum	Luteal
No of ova examined*	41	133	404
With vesicular nucleus	11	87	232
Prometaphase I	4	17	42
Metaphase I	14	14	77
Anaphase I	2	1	5
Telophase I	0	1	4
Metaphase II	8	13	40
Pycnotic	2	0	4

* Ova from follicles 3 mm in diameter and larger

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Ova from follicles 3 mm in diameter and larger

yet unovulated follicles is unknown, but it is surmised that they would beco atretic and subsequently degenerated This would eliminate them from futi reproductive processes and to a certain extent temporarily deplete the ov of many of the tertiary follicles This may account for the condition descril by Willet (1952) as ovarian refractiveness in repeated administration gonadotropins for producing superovulation in cows

Fertility of Induced Ovulated Ova

Attempts were made to fertilize these eggs by breeding some of the anim Of the 45 eggs (5 from proestrous 7 from anestrous and 40 from luteal ew examined from bred animals only three showed cleavage Two of these f tilized eggs were from proestrous ewes and one from a ewe that had previou been in anestrum

Sperm were never seen in the zona pellucida of the non fertile eggs T would tend to indicate that either the zona was impenetrable by the sperm else adequate number of sperm never reached the upper region of the ovid to effect fertilization The latter seems most likely since recovery of spe from the washings of the uterus and oviduct was very limited It is belie that the tract and especially the uterus is unfavorable for sperm survival wh a corpus luteum is present This is further substantiated by the fact that in luteal ewes that were bred by injecting sperm directly into the horn of t uterus all exhibited a very large number of leucocytes and phagocytes in t lumens of the uterus within 40 hours

Cervical Smears

Rowland (1952) and de Paz (1953) reported that at times the cervical secretic are hostile to sperm survival They noted a definite relationship between t hostility and low blood estrogen level These workers observed that smears cervical secretions that are receptive to sperm survival exhibit a fernlike p tern when flame dried and examined under the microscope A smear of t type was designated as typical Smears of cervical secretions that are hosl to sperm survival are characterized by the absence of a fernlike pattern wh flame dried They designated smears of this type as negative Atypical was t term used to denote the condition between negative and typical

The cervical secretions of ewes in estrus and for at least one day followi estrus gave a fern like pattern when flame dried (FIG 3g) Smears ma from ewes which were treated with stilbestrol in combination with gonad tropins most often gave a negative smear (FIG 3i) Occasionally atypical response was obtained (FIG 3h) Some of the ewes yielded occasional smear which was classed as typical However within 48 hours aft injection the typical smear had reverted to a negative pattern With the lev of hormones used (35 Armour Units of gonadotropins and 12-15 mg stilbestrol) the cervical smears were predominately negative

From this study it is apparent that the eggs which were induced to ovulate with gonadotropins are normal in so far as the maturation process is concerned It is believed that the hostility of the female tract toward sperm longevity is the major obstacle preventing fertilization

SUMMARY

The maturation process of normal and artificially ovulated ova of ewes was studied cytologically

Normally the first maturation process of the ovum nucleus is completed during estrus Metaphase I stage is evident by mid-estrus and the first division is completed before the expulsion of the ovum from the follicle Just prior to ovulation the first polar body is formed

Induced follicular growth maturation and ovulation were obtained by intramuscular injections of Armour s pituitary gonadotropin (FSH)

Artificially ovulated ova undergo the normal process of maturation including the extrusion of the first polar body and the formation of a viscous plug around the egg prior to ovulation

The shortest lived structure of the matured female germ cell appears to be the chromatic material for this is the first unit to show visible degenerations when fertilization does not occur This degeneration is manifest by a break down of the maturation spindle and a random scattering of the chromosomes into the adjacent cytoplasm

The eggs obtained from the luteal ewes that were bred gave no evidence of fertilization It is assumed that the reproductive tract of these ewes was not favorable for adequate sperm survival since no sperm were observed in the zona of the egg and only a very few sperm if any were found in washings from the tract

Cervical secretion smears made from the animals treated with gonadotropic hormone alone were negative possibly indicating that their cervices were hostile to sperm longevity This condition was somewhat changed by injections of stilbestrol given in combination with the gonadotropin as indicated by the presence of the fernlike pattern for a short interval of time in some of the smears of treated ewes

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RELATION OF THE NERVOUS SYSTEM TO IMPLANTATION*

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University of Illinois

IN A series of publications from this laboratory (Moore and Nalbandov, 1953 1955, Nalbandov Moore and Norton 1955) it was shown that uterine contents exert a profound effect on the duration of functional activity of corpora lutea and that prolactin has luteotrophic effect in sheep In brief the experiments consisted of implanting plastic beads into the cornu of normally cycling ewes in different stages of the estrus cycle When a bead was implanted on day 3 of the cycle subsequent cycles became significantly shortened (Table I) Removal of the beads caused the return of cycle length to normal Subsequent experiments showed that implantation of the bead on day 8 of the cycle caused an increase in cycle length and that the bead had to be larger than 2 mm in diameter to cause a lengthening or a shortening of cycles (Table II) Furthermore it was shown that denervation of the horn segment containing the bead prevented modification of cycle length (Table II) The types of operation performed and the degree of distention obtained is shown in FIG 1 of Moore and Nalbandov 1953

These results were interpreted to mean that uterine contents—beads and hence probably embryos—are capable of determining the length of survival of corpora lutea Since denervation of the uterine segment containing the bead prevented modification of cycle length the theory seemed plausible that under certain circumstances a neural stimulus emanating from the uterine lumen

TABLE I

Effect of Presence or Absence of Beads in Uterine Cornu on Length of Estrous Cycle

Animal No	First cycle length (days)	Cycle length with bead in uterus (days)	Cycle length bead removed from uterus (days)
913	16	10 5 5 5 9	15 7 15
378	16	7 15 5	16 16 18
858	17	6 14 7	15 16 17
Mean	16 33	8 18	16 11

* This work was supported by grants from USPH (No 3557C through 4C)

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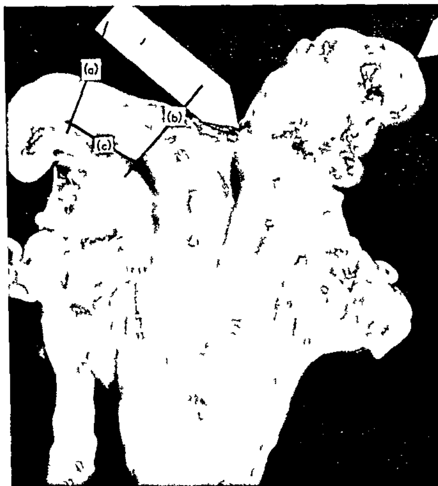


FIG 1 Black lines on left cornu show the cuts made to denervate a uterine segment The arrows on right horn point to the actual cuts made in a uterine cornu containing a 12 mm plastic bead located in the upper third of the denervated segment

TABLE II

Effect of Uterine Distention on the Estrous Cycle Length of the Ewe

Day of cycle	Diameter of bead (mm)	Number of ewes and cycles	Mean cycle length (days)
3	2	5–21	16 7±0 42 SE
	4	4–8	12.8±2 08
	8	4–22	13 0±1 30
8	2	8–30	17 3±0 49
	8	8–22	22 8±1 21**
	8 + den	5–18	17 0±0 55
13	2	7–16	16 1±1 00
	8	7–27	17 8±0 80
—	—	48–61	16 3±0 11

SE = Standard error of the mean * = P 0 05 ** = P 0 01

notifies the pituitary gland via the hypothalamus that the corpus luteum should be maintained The fact that beads were able to prolong cycles to a maximum of only 23 days (vs the normal length of 16 days) is not considered particularly disturbing The bead after all is an inert object of fixed size which does not enter into intimate relationship with the uterine endometrium while the growing blastocyst elongates enormously during the early stages of pregnancy and eventually becomes intimately attached to the uterine wall It is obvious that living and growing uterine contents provide a much more adequate and sustained neural stimulus than can be provided by the bead

The next step in these experiments was to determine the pathways by which the stimulus from the uterus was translated into the presumed continued production of luteotrophic hormone by the hypophysis The present paper is to be regarded as a preliminary report of this work Sections of the hypophyseal stalk were studied as the next step in these experiments The results obtained very soon confirmed the finding of Harris (1955) and others on rats who established that stalk section to all intents and purposes was equivalent to hypophysectomy if the cut stalk ends were prevented from re establishing contact and from regenerating However during the early stages of experiments on sheep it was not known in which of these operations stalk regeneration was successfully prevented The majority of animals in which this operation was attempted continued to cycle normally and it was found at autopsy that in them the stalks were cut incompletely or that they had re established neural and humoral connections post operatively Some of the animals with cut stalks were mated to fertile rams prior to autopsy but only a very small number of them conceived There appeared to be a relationship between the complete

ness of the stalk section and the ability of these animals to conceive and suggested the possibility that even though enough of the stalk connections remained intact to insure normal cyclic behavior the damage to the pathway was sufficiently great to interfere with the ability of these animals to conceive

Concurrently with the experiments on the sectioning of the hypophyseal stalk studies were made on the effects of cutting the hypogastric nerves supplying the uterus This experiment at first glance proved disappointing because all the females so treated continued to have cycles of normal length and to ovulate at the expected time However the unexpected happened when females in which the stalks or the uterine nerves had been cut were mated to fertile males Matings were begun in November and continued until March 1956 The results obtained in both groups of sheep are shown in Table III Because of the similarity of results obtained in the two groups it was felt that combining the groups and comparing their breeding efficiency to a control group of normal females was justified (bottom lines Table III) Attention is called to the fact that when the stalk is completely severed and when it is prevented from regenerating all reproductive activity stops and the reproductive organs become atrophic Incomplete stalk section or section of the uterine nerves has no effect on cyclic reproduction behavior but 50% of the animals in which these operations were performed did not conceive after an aver

TABLE III

Effect of Section of Hypophyseal Stalk or Hypogastric Uterine Nerves on Reproductive Performance

Treatment	Number of females	Total number of times bred	Average number of cycles bred before autopsy	Reproductive state at autopsy
Stalk cut	2	7	3.5	2 with fertilized eggs
Stalk cut	1	not bred	—	normal reprod organs
Stalk cut	3	not bred	—	no heats reprod organs atrophic
Stalk cut	5	11	2.5	pregnant
Uterine nerves cut	6	21	3.5	4 with fertilized 2 with uncleaved eggs
	1	not bred	—	no heats after operation
	3	6	2.0	pregnant
Combined not pregnant	8	28	3.5	6 with fertilized 2 with uncleaved eggs
Combined pregnant	8	17	2.13	pregnant
Normal controls	10	12	1.2	pregnant

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age of 3½ breedings per female Of significance is the fact that in 6 of the 8 animals in this group fertilized and cleaved ova were recovered at autopsy while in two of the ewes the eggs recovered were uncleaved with sperm being seen in the perivitteline space Only 3 out of 9 animals with severed uterine nerves conceived during the time these females were exposed to fertile males and these females required two matings per conception It is of course, unknown whether all of them would have conceived prior to the termination of the breeding season had matings continued, but it appears significant that even those females which did conceive required about twice as many matings as did their unoperated controls

DISCUSSION

The reasons for the failure of the females in which the nerve connections between the uterus and the pituitary gland is interrupted to conceive to a normal number of matings remain to be determined It is possible that following nerve section the signal (which is thought to emanate from the uterus) for maintenance of corpora lutea fails to reach the pituitary gland or reaches it less reliably than it does in intact females If this assumption which forms the basis for reasearch now in progress is correct it may adequately explain the phenomena described It is also interesting to note that the reproductive behavior of these females resembles the reproductive pattern of so-called hard to settle females which show estrous cycles of normal length normal time and rate of ovulation and fertilization but which fail to conceive in spite of repeated matings The question arises whether in these cases there is an impairment of the signalling mechanism existing between the uterus and the hypophysis leading to non maintenance of the corpus luteum and hence to failure of implantation That in these cases too the impairment is not necessarily permanent can be seen from the fact that hard to-settle females conceive eventually if they are mated sufficiently frequently

SUMMARY

The nerve connection between the uterus and the hypophysis can be interrupted by cutting the hypophyseal stalk or by cutting the uterine splanchnic nerves

Incomplete stalk section or cutting of uterine nerves has no effect on normal heats ovulation and fertilization but animals so treated either fail to conceive in spite of repeated matings to fertile males and in spite of the fact that the majority of eggs ovulated are fertilized Those conceiving require significantly more breedings than do normal controls

These results are interpreted to mean that the cutting of the nerve connection between the hypophysis and uterus prevents a neural signal emanating from the latter to reach the pituitary gland and to cause it to secrete the luteotrophic hormone necessary for maintenance of corpora lutea during pregnancy

pora lutea between 25 and 45 days of pregnancy and stressed the variation in size shape and staining qualities of the individual lutein cells During the second month of pregnancy this investigation showed that the lutein tissue was more compact and the lutein cells were larger and more rounding in shape with the cytoplasm being more lightly stained than between 25 and 30 days

More comprehensive investigations have been conducted on the corpora lutea of other species Corner (1915 1919) in his classic investigations of the corpora lutea of pregnancy in swine described three principal types of lutein cells (1) true lutein cells originating from the granulosa (2) type 1 cells with smaller round or oval and more chromatic nuclei which occur on the periphery of the corpus luteum and along the connective tissue septa (3) type 2 cells with a spindle shape and a cytoplasm that stained dark brown or purple with Mallory s stain He noted also that there were many cells which seemed to be transitional stages among all three types Warbritton (1934) concluded from a detailed histological study of the cyclic corpus luteum in the ewe that the three types of cells which were present (embryonic normal regressing) repre ented three phases in the life cycle of a single lutein cell derived from the granulosa

Brewer (1942) after an intensive study of human corpora lutea of menstruation was able to estimate their age within two or three days on the basis of their histological characteristics Among the signs of regression or degeneration of granulosa lutein cells Brewer listed the following (1) a shrinkage in over all cell size (2) an increase in the size and number of lipoid droplets (3) vacuolated cytoplasm (4) dense shrunken and pycnotic nuclei (5) increased connective tissue (6) infiltration by leucocytes Gillman and Stein (1941) described in detail specific inclusions of the granulosa lutein cells in the human corpus luteum of pregnancy including secretory granules lipoid substances colloid droplets vacuoles chromidial substance mitochondria and Golgi apparatus They reported that secretory granules lipoid droplets vacuoles and chromidial substances were numerous in the granulosa lutein cells during the first half of the gestation period and tended to decrease with advancing gestation Colloid droplets on the other hand were rare during the first two months of pregnancy but 50% of the cells contained colloid at term These authors also described light and dark cells and concluded that they represented different phases of secretory activity and that the dark lutein cells eventually degenerated White *et al* (1951) studied 28 corpora lutea of normal human pregnancies from the two celled egg stage up to 4½ months of pregnancy and 13 corpora lutea associated with abnormal ova They described a cell type identified as K which was characterized by a stellate shape eosinophilic cytoplasm and a small dense hyperchromatic nucleus which was irregular in outline These investigators found a direct relationship between the amount of trophoblast associated with implanted abnormal ova and the

CYTOLOGICAL CHANGES IN THE BOVINE CORPUS LUTEUM DURING EARLY PREGNANCY*

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REPRODUCTIVE failures in both beef and dairy cattle present major problems in herd management and disease control to beef producers and dairy farmers throughout the United States Until recently very few data based upon fundamental research have been available for the purpose of evaluating the importance of the bovine ovaries the uterus the placenta and the embryo itself as factors in lowered reproductive efficiency resulting from functional causes A thorough understanding of the morphological characteristics of the functional corpus luteum of pregnancy is essential to the recognition of primary or secondary luteal failures associated with early embryonic mortality

REVIEW OF LITERATURE

Notwithstanding the importance of the corpus luteum in cattle for establishing and maintaining a normal pregnancy the information in the literature relative to its histological characteristics during early pregnancy is very limited Furthermore in many cases the previous breeding history of the cattle studied was unknown or not reported Hammond (1927) reported that the corpus luteum of pregnancy in cattle resembled that of mid cycle in microscopic appearance McNutt (1924 1927) studied the cyclic corpus luteum as well as the corpus luteum of pregnancy in the ox and concluded that the lutein cells arise from both the granulosa and theca interna but added that many lutein cells exhibited intermediate characteristics and the origin of these cells could not be stated with certainty He concluded also that regression of the corpus luteum of pregnancy did not differ in any way from regression of the cyclic lutein cells Asdell (1946) stated that the corpus luteum reached its greatest size about the fourth month of pregnancy and that regressive changes began a little before parturition but that more careful study was needed Foley and Reece (1953) reported on the gross and microscopic anatomy of bovine cor

* Supported by th North ast Cooperative regional project NE 1 Causes and Prevention of Reproductive Failures in Cattle and by funds from Dairy Husbandry Research Branch USDA Beltsville Maryland

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segments The appearance of the lutein tissue especially in younger corpora lutea is quite variable both along a radius from the central area toward the periphery and along an axis from the ovulation papilla to the opposite pole The individual lutein cells are surrounded by a thin layer of connective tissue and capillaries are abundant throughout the tissue in close association with each cell Some leucocytes are usually present

Earlier reports to the contrary it seemed logical to expect the cytological appearance of the corpus luteum to change with advancing pregnancy and to reflect any abnormal developments in the early bovine placenta and embryo The sections of corpora lutea from each individual cow or heifer were there fore studied in detail in an effort to record such changes Since the youngest tissues in this study represented 16 days of gestation no attempt was made to identify the lutein cells on the basis of origin For our purposes whether they came originally from the granulosa or the theca interna was of less concern than their appearance and function during the period under investigation

It was evident at once that the individual lutein cells exhibited marked dif ferences in size shape and appearance of the nuclei and in staining qualities and vacuolization of the cytoplasm After studying representative sections of tissue from cattle slaughtered at each day of pregnancy between 16 and 33 days the authors were able to estimate within a day or two the stage of gestation of unmarked tissues Further study showed that the various types of lutein cells could be classified into five groups In this investigation the lutein cells will be designated as Types I II III IV and V but they are considered to represent different stages of growth regression or secretory activity of a single basic lutein cell It was also possible to identify unmarked sections of corpora lutea as being from cattle with normal reproductive histories (Group I) or from abnormal cattle (Group II) on the basis of the proportion of the five lutein cell types the amount of connective tissue the degree of vascularization of the lutein tissue and the vacuolization of the cytoplasm of the lutein cells The cytological characteristics of each of these five cell types are described in the following paragraphs

TABLE I

Mean Diameters of Five Lutein Cell Types and Their Nuclei

Type	Size of cells Diameters in micra Width Height	Size of nuclei Diameters in micra Width Height
I	10 5±4 0 × 18 0±5 5*	5 5±1 5 × 6 5±1 0
II	18 5±4 5 × 27 5±7 0	7 0±1 0 × 9 0±1 5
III	15 0±4 0 × 26 5±6 0	6 0±1 0 × 8 0±1 0
IV	13 0±4 0 × 31 0±8 5	5 0±2 0 × 7 5±1 5
V	7 0±2 5 × 16 5±4 0	5 0±0 5 × 6 0±1 5

* Standard deviation

appearance of the corpus luteum Embryos almost devoid of trophoblast were without exception accompanied by corpora lutea characterized by total uniform colloid degeneration of all K cells

MATERIAL AND METHODS

One hundred and fifty six head of dairy and beef cattle have been slaughtered in this project On the basis of their previous breeding history and ante and post mortem examination of their reproductive tracts 76 head were classified in Group I (normal) On the same basis 32 other cows and heifers were classified in Group II (abnormal) Forty eight cattle were classified in Group III (doubtful) These cattle were abnormal in some respect Some required more than two services others were abnormal in one way or another upon post mortem examination though the previous breeding history may have been normal Of the 93 cattle which were pregnant when slaughtered 56 head yielded timed embryos between 16 and 33 days of gestation The others were culled at more advanced stages of pregnancy

The corpora lutea from all individuals were weighed sub sampled and fixed in Bouin s formol sublimate or buffered neutral formalin Tissuemat sections fixed in Bouin s were stained with hematoxylin–eosin and Mallory s connective tissue stain Sections from formol sublimate fixed tissue were stained with picro Mallory phloxine–methylene blue (Greenstein 1957) and periodic acid–Schiff (PAS) stains Frozen sections of gelatin embedded tissues fixed in neutral formalin were stained for lipids with Oil red O and Sudan black

In this investigation the lutein cells were classified for descriptive purposes, into five types designated by Roman numerals and the data in Table I were obtained by measuring 25 representative cells of each type To obtain the per centage distribution of cell types shown in Table II, 500 individual lutein cells were counted in each section using a 6 mm² eyepiece crossline micrometer at ×512 magnification Twenty five cells were counted at each of 20 sample areas selected at random in the section of tissue Six areas were selected in a central vertical plane and fourteen additional counts were made diagonally across the long axis of the section The cells to be counted were selected at the intersections of the vertical and horizontal lines of the grid in the upper left and lower right quarters Counts were made from slides with tissue identifica tion numbers masked out Only lutein cells which could be positively identified were counted

RESULTS AND DISCUSSION

The corpus luteum of pregnancy in cattle is an eccentric oval body varying in color from a deep yellow through various shades of orange to a cocoa brown and ranging in weight in this investigation from 3 20 to 9 30 g The outer connective tissue capsule is well vascularized and trabeculae with abundant fibroblasts grow inward towards the center dividing the tissue into well defined

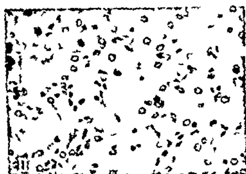


Fig 1 Bovine *corpus luteum* on sixte nth day of normal pregnancy NE .0 Group I Mostly Type I and II lutein cells a few III s and IV s This is quite typical of the lutein tissues included in Group I in Table . Hematoxylin and eosin ?10



Fig ? Lutein cells on 19th day of normal pregnancy NE 28 Group I Typical Type II blossom cell at upper right Type I cells at left Mallory s triple stain 750



Fig 3 Lutein tissue on 21st day of normal pregnancy NE-43 Group I Type III cell in center with shrunken granular cytoplasm and crenated nuclear membrane Note Type IV cells at upper left and lower right and fibroblasts in surrounding connective tissue Picro-Mallory 750

Type I Cells

Type I cells are quite variable in size and shape but are easily identified by their round centrally located vesicular nuclei with one or more prominent nucleoli These cells are less plump and regular in cell outline than Type II cells and as shown in Table I vary in size from one third to one half of the Type II cells The nuclei are about two thirds the size of Type II nuclei The cytoplasm is lightly stained but not as pale staining as some Type II cells These immature lutein cells are present in all corpora lutea of early pregnancy in substantial numbers (FIGS 1 and 2) In corpora lutea from normal cattle they apparently grow and become mature Type II lutein cells

Type II Cells

These mature lutein cells often referred to as blossoms in the literature are the largest lutein cells of the five types and exhibit a large round or slightly oval vesicular centrally located nucleus with a distinct nuclear membrane one or more prominent nucleoli plus limited amounts of other chromatin material surrounded by a clear nuclear sap These plump cells round or *slightly oval in shape* with a distinct cell wall and lightly stained granular cytoplasm are a characteristic feature of normal corpora lutea during early pregnancy (FIGS 1 and 2) As indicated earlier they are quite similar to the Type I cells except for larger size Cells which are intermediate between a typical Type I and Type II cell can be seen in all sections (FIG 1)

Type III Cells

Type III cells are intermediate in appearance between Type II and Type IV cells Both the cell wall and the nuclear membrane appear to have shrunken This frequently leaves empty spaces between the cell and its neighbors The nucleus irregular in outline is coarsely granular in contrast to the vesicular nuclei of Type II cells The cytoplasm stains more intensely than Type II cytoplasm and the cell outline tends to be elipsoidal (FIG 3) The Type III cells are quite variable in shape and staining qualities and sometimes the cell wall is indistinct or completely disintegrated It is believed that these cells represent the initial stages of regression which leads to Type IV and Type V cells

Type IV Cells

These cells are easily identified even at low magnifications by the intense and diffuse staining of the cytoplasm with various types of stains by their stellate or spindle shaped appearance and by their hyperchromatic nuclei The typical cell is quite attenuated often vacuolated and possesses one or more cytoplamic processes The nucleus is oval to spindle shaped with variable and progressive loss of nuclear detail characteristic of pycnosis (FIGS 3 and 5) Their distribution is highly irregular both from tissue to tissue and among areas in the same tissue If these cells do not represent a regressive phase from

the Type II cells they certainly indicate a marked change in secretory activity or some other aspect of cellular physiology

Type V Cells

The smallest lutein cells measure 7 0 ±2 5 × 16 5 ±4 0 μ in diameter as shown in Table I with a small dense nucleus measuring 5 0 ±0 5 × 6 0 ±1 5 micra These amorphous cells with pycnotic nuclei and very limited cyto plasm are relatively rare in the tissue but are believed to represent the end point of the series of regressive changes in the lutein cells

Changes in Normal Corpora Lutea with Advancing Gestation

Asdell (1955) states that the cyclic corpus luteum begins to regress about the sixteenth day following an estrous period Unfortunately our series of cyclic corpora lutea from normal cattle has not been completed and it is not yet possible to compare the morphology of a 16 day cyclic corpus luteum with that from an animal bred during the previous estrous period However the changes which occur in corpora lutea from normal cattle slaughtered between 16 and 33 days of pregnancy can be described

At 16 days of pregnancy the lutein tissue is reasonably compact and exhi bits a well developed capillary network around the lutein cells Type I and Type II lutein cells predominate but III s IV s and even V s can occasionally be seen (Fig 1) Type I cells are much more numerous than Type II cells and the latter are smaller and less plump than at later stages Type III cells when present show the characteristic shrunken appearance described earlier and when the tissue is stained with Mallory s connective tissue stain their nuclei are often extremely fuchsinophilic

Between 17 and 22 days of pregnancy the corpora lutea of Group I cattle with normal breeding histories exhibit considerable variation in their cyto logical appearance Type I and Type II cells predominate but their relative proportion varies and most of the Type II cells have not reached their maxi mum size Type III cells are more common at this stage than at 16 days especially in some tissues Type IV and V cells are present in scattered areas and in limited numbers As a result it is more difficult to estimate the exact number of days of gestation during this period

From 23 to 33 days of gestation the presence of many large plump Type II blossom lutein cells makes it easy to recognize tissues from cattle slaugh tered during the fourth week of normal pregnancy The lutein tissue is firm and compact with a minimum of space between the individual cells When stained with Mallory s connective tissue stain Type V cells continue to show fuchsinophilic nuclei and occasionally a Type II or III cell exhibits brilliant pink secretory granules throughout the cytoplasm The significance of these granules is not understood Some lutein cells are binucleate and a limited number of leucocytes are scattered throughout the tissue



FIG 4 Lutein tissue averaging 45 6% Type III cells at 23 days of gestation NE 113 Group III (See Table II) Note 3 Type III cells open tissue structure and numerous fibroblasts and leucocytes Picro-Mallory 750



FIG 5 Three Type IV cells at right Type I cells on left and portion of Type II cell at bottom NE 19 Group III 22 days post breeding Note extreme vacuoliza tion of Type IV cell at upper right and characteristic cytoplasmic processes dense cytoplasm and pycnotic nuclei of other IV s This Group III heifer yielded chorion but no embryo when slaughtered at 22 days and the corpus luteum is be ginning to regress Picro-Mallory × 750

FIG 6 A regressing corpus luteum at 27 days post breeding NE-44 Group II Note arteriole and numerous capill aries excessive connective tissue vacuoles in coarsely granular cytoplasm of scat tered lutein cells with pycnotic nuclei and empty spaces left by lutein cells resorbed or phagocytized by leucocytes This cor pus luteum was small 2 ˌ0 g and heavily encapsulated when recovered Picro Mallory 300

TABLE II

Percentage Distribution of Preliminary Cell Counts in Normal and Abnormal Corpora Lutea During Early Pregnancy

NE Number	Number Services	Days Post Breeding	Percentages of Cell Types I and II	III	IV and V	Pregnant
Group I—normal						
NE25	2	18	88 0	4 6	7 4	+
NE37	1	18	74 6	11 2	14 2	+
NE66	1	20	80 4	13 2	6 4	+
NE81	1	2_	85 0	12 4	2 6	+
NE48	1	23	85 4	9 8	4 8	+
NE6	1	26	93 0	5 0	2 0	+
NE24	1	_8	74 6	18 2	7 2	+
AVERAGES	1 1	22 1	83 0	10 6	6 4	
Group III—doubtful						
NE58	2	20	55 6	27 6	16 8	–
NE62	1	20	45 2	27 6	26 8	–
NE113	1	23	23 4	45 6	31 0	+
NE119	2	29	19 4	56 2	24 4	–
AVERAGES	1 5	23	35 9	39 2	24 7	
Group II—abnormal						
NE68	5	18	34 8	34 8	30 4	–
NE63	9	20	9 2	52 4	38 4	Ch *
AVERAGES	7	19	22 0	43 6	34 4	

Chorion only

Among the Group III cattle neither NE 58 nor NE 62 yielded a timed embryo when slaughtered at 20 days and the cell counts shown in Table II indicate that both corpora lutea were beginning to regress NE 113 classified in Group III because of a previous abortion showed only 23 4% normal lutein cells at 23 days but was pregnant when slaughtered NE 119 was open at 29 days though she passed over one estrous period Both horns were filled with cream colored pus The tissues from these two animals average 55 9% III and about 27 7% IV and V

The corpus luteum of NE 68 (Group II) was definitely regressing when the animal was slaughtered at 18 days but this could have been either nonfertiliza tion or resorption of the blastocyst prior to slaughter NE 63 (Group II) yielded chorionic tissue proving fertilization but the embryonic disk was not located in the knotted chorion upon subsequent sectioning This corpus luteum was obviously in an advanced stage of regression at 20 days and this is supported by the cell counts

Corpora Lutea from Group II and Group III Cattle

Corpora lutea from cattle classified in Group II (abnormal) and Group III (doubtful) could not be distinguished in most cases from those of normal cattle on the basis of size shape color weight or other gross morphological characteristics Weight data on corpora lutea from all three groups are being analyzed statistically in relation to the weights of ovaries and the live weight of the cattle

Nineteen cattle yielded corpora lutea with fluid filled central cavities of varying size Three were classified in Group I eleven in Group II and five in Group III These data also are being studied in relation to the histological characteristics of the corpora lutea

As indicated earlier it was possible in most cases in this investigation to identify tissues from cattle classified in Group I (normal) and Group II (abnormal)

It should be emphasized however that the accuracy of the identification depends upon the amount of regression which has occurred in the corpus luteum While the proportion of the five cell types in the lutein tissue especially the relative numbers of Type II and IV cells is a major factor in classifying the tissue it must be understood that other characteristics also contribute to the final decision and it may not be possible or desirable to evaluate the tissues strictly on a mathematical basis Other criteria which must be considered are the compactness of the tissue the degree of vacuolization the amount of connective tissue including the number of fibroblasts present the size and distribution of smaller blood vessels and capillaries the number of leucocytes and the appearance of the cytoplasm and the nuclei under various types of stains

Nevertheless in an attempt to obtain a more objective method of evaluating the lutein tissues preliminary cell counts have been made on sections selected at random and the results are summarized in Table II Since both Type I and Type II cells are associated with normal development and function of the corpus luteum they are combined in a single figure in the table Likewise Types IV and V cells are shown as a single figure Type III cells which represent an intermediate stage are shown separately

The average percentages shown in Table II for the Group I tissues support the earlier general observations relative to the appearance of the lutein cells during early pregnancy All of these cattle yielded normal embryos when slaughtered and the corpora lutea were characterized by a high percentage of Type I and II cells and with one exception by a low percentage of Type IV and V cells With only seven animals represented here the effect of advancing gestation on cell type distribution is not clearly demonstrated The greatest variation percentage wise occurs in the Type III cells and this in turn affects the percentage of Type I and II cells

OVARIAN FUNCTION, BLOOD BIOCHEMISTRY AND REPRODUCTIVE PERFORMANCE OF REPEAT BREEDER COWS*

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Repeat breeder cows comprise a group of cattle which has been studied intensively by numerous research teams and veterinary practitioners From their investigations of the various aspects of functional sterility has been compiled a wealth of statistical information however the basic cause or causes of the syndrome has successfully evaded elucidation

Many reviews have been ably presented on the various causes and treatment of repeat breeding therefore this report is only intended as a supplement to the available data already published

The Michigan Artificial Breeders Cooperative in co operation with the Michigan Agricultural Experiment Station initiated a study of repeat breeder cows in this state This project was undertaken to obtain information which could later be utilized to guide more intensive research on the problems found to be most urgent under field conditions

METHODS AND PROCEDURES

A total of 50 repeat breeder cows and heifers was obtained from local dairy men Each animal was examined by a member of the project to establish its previous breeding history and present health status Only those cows were purchased which (*a*) were negative to the agglutination test for brucellosis vibriosis and leptospirosis (*b*) had been unsuccessfully bred at least four times (*c*) were between the ages of 2 5–10 years and (*d*) revealed no evident cause for sterility upon physical and bacteriological examination All cows were non lactating or were dried upon entrance to the experiment Grade and purebred cows and heifers of the Holstein Guernsey and Jersey breeds were included All cows were given a rest from breeding for a minimum of one heat period

Approved for publication by the Director of the Michigan Agricultural Experiment Station as journal article no 2106

This work was supported in part by a grant from the Michigan Artificial Breeders Cooperative

Though the number of lutein cell counts completed to date is limited the data acquired thus far suggests that this approach may be useful in recognizing luteal failures during early pregnancy The average percentages for Groups I II and III in Table II are consistent with the earlier general impressions gathered from these tissues

Summary and Conclusions

1 The lutein cells of bovine corpora lutea recovered from cattle slaughtered between 16 and 33 days of gestation can be grouped into five types on the basis of their cytological characteristics

2 Type I cells represent immature lutein cells and Type II cells are mature cells which have reached their maximum size and development

3 Type III cells are believed to be in the initial stage of regression which continues through Type IV cells and terminates with Type V cells

4 Between 16 and 33 days of gestation there is an increase in the number and the size of the Type II cells with a corresponding reduction in I s

5 Lutein tissues with high percentages of Type III IV and V cells especially Type IV cells are considered to be abnormal

6 The cytological changes in the bovine corpus luteum during early pregnancy coincide with advancing gestation and are related to the reproductive performance of the individual animal

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recovered from cows going to slaughter from the experiment These were observed for gross lesions or abnormalities photographed and tissues obtained for histopathological examination The ovaries were observed grossly for evidence of ovulation and in 12 of the cows slaughtered 24–72 hours after insemination the oviducts were flushed with physiological saline The ova thus obtained were observed for fertilization

RESULTS

Data on 50 repeat breeder cows and heifers were obtained over a 6 year period During this time a total of 32 cows and heifers conceived This represents 70% of the total numbers These figures exclude four animals which were pregnant on arrival Seventy nine per cent of the 28 cows conceived after an average interval of 10 8 months and 46% of the 22 heifers conceived after an average interval of 12 3 months A comparison between the cows and heifers on the control and experimental rations indicates a 72% conception rate for control animals and a 65% conception rate for those on the experimental rations

From accurate records available it was determined that 21 cows which conceived required an average of nine inseminations per conception and ten heifers which conceived required an average of 9 6 inseminations per conception

These figures represent the interval from first insemination until eventual conception and include the time the animals were in the owners herds plus the time on experiment

In contrast to these data it is noted in Table I that following entry into the experiment the period of time until conception and inseminations per conception are reduced in both control and experimental groups It is interesting to note that a total of 17 cows and heifers of the 32 which conceived did so after only one insemination following entry into the experiment

TABLE I

Inseminations per Conception and Period of Time in Experiment until Conception

	Insemination per conception	Interval to conception
Combined average	2 5	2 93 (months)
Cows	2.14	2 75 (months)
Heifers	3 09	3 27 (months)

The average interval in days of the estrous cycle was determined from data obtained in the experiment plus any reliable data from the farm breeding records The cycles which fell within the range of obvious double cycles or

between last farm insemination and first insemination on experiment to establish their normal estrus cycle

The cows were alternately placed in a control or experimental group as they entered the experimental herd Those in the control group received an average Michigan farm ration consisting of average to low protein mixed hay a 16% crude protein grain mixture of corn oats soybean oil meal 3% steamed bone meal and 3% salt The cows in the experimental group received a ration consisting of second cutting alfalfa hay containing 14–16% crude protein with a minimum of 10 µg of carotene per gram and a 16% crude protein grain mixture of corn oats wheat bran soybean oil meal 3% steamed bone meal 3% salt and all the known trace minerals

Feed and blood samples were obtained and analyzed periodically for various constituents by the Department of Agricultural Chemistry

During the months of May through October both groups grazed the same permanent pasture consisting principally of blue grass and smooth brome No fertilization of the pasture was carried out nor were pasture plants and grasses analyzed for constituents

Cows and heifers were checked twice daily for onset or termination of estrus between 8 a m and 5 p m Onset or termination of estrus occurring between 5 p m and 8 a m was arbitrarily determined as one half the time between check periods In some instances the length of estrus was more closely determined concurrently with a series of bi hourly ovulation examinations Estrus was considered to be standing heat and it was considered terminated when the cow no longer stood to be mounted Except in the early stages of the experiment a nymphomaniac cow was maintained as a tease animal for the detection of estrus

All cows in the experiment were inseminated artificially by a member of the project The semen was obtained from the Michigan Artificial Breeders Co operative and was the same as that provided for use in dairy herds throughout the state None of the semen was more than 48 hours old when used

The cows in the control group were transferred to the experimental group if after six months they had not conceived

The cows in the experimental group were inseminated on two successive estrus periods and if they failed to conceive were treated with various hormone preparations as dictated by their endocrine function These included thyroprotein chorionic gonadotropins oxytocin and progesterone

In general those cows failing to conceive after a year in the experimental group were considered sterile and subsequently sent to slaughter

Except in two instances all cows which conceived and were not returned to the owners were maintained in the herd until parturition Gestation and parturition data on the pregnant cows returned to the owners were obtained whenever possible but subsequent conception data were not obtained

The reproductive tract thyroid adrenal and some pituitary glands were

establish the fact of ovulation in each cow and heifer In only three observed heat periods was there reasonable assurance of ovulation failure and one of these animals eventually conceived All animals which were sent to slaughter displayed evidence of recent ovulation and luteal development

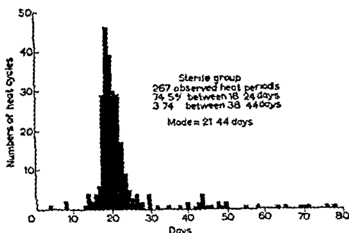


FIG 2. Histogram showing distribution of estrous cycles in the sterile group

Table III gives the results of attempts to recover ova from cows and heifers on post mortem examination Six ova were recovered of which only two were in the eight-cell stage This would suggest that only two ova had been fertilized in this group In six other attempts no ova were recovered and one can only speculate whether this failure was due to faulty technique or the absence of ova in the Fallopian tubes

TABLE III

Post mortem Recovery of Tubal Ova

Recovered	*Not recovered*
2 ova in 8 cell stage	2 animals showed hydrosalpinx
4 ova unfertilized	4 animals grossly normal
All animals had ovulated	

Upon post mortem examination 16 of the cows and heifers showed gross lesions or abnormalities involving the reproductive tract which might have affected fertility These abnormalities included hydrosalpinx myometrial cysts ovarian adhesions ovarian cysts and cervical stricture Included in this group were 6 cows and 1 heifer which had conceived while on the experiment The most consistent abnormality noted among the cows and heifer was

greater have been eliminated because of the difficulty in determining whether or not the long periods of anestrum were real or presumed The means are outlined in Table II

TABLE II
Interval (days) of Estrous Cycle

Group	No of cows	Average
Fertile	25	21 59
Sterile	13	21 16

To determine more accurately the period of greatest frequency of the average estrus cycle histograms were constructed for the fertile and sterile groups Figure 1 indicates that in the fertile group 72 2% of the estrous cycles fell within the 18–24 day interval with a mode of 21 67 days whereas in the sterile

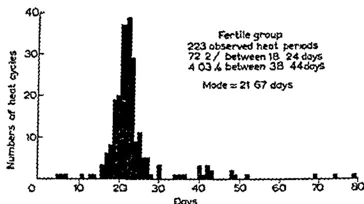


FIG 1 Histogram showing distribution of estrous cycles in the fertile group

group (FIG 2) 74 5% of the observed estrous cycles occurred within the 18–24 day interval with a mode of 21 44 days Asdell de Alba and Roberts (1949) determined the mode of estrous cycle length in normal heifers as occurring at 20 days and in normal cows at 21 days This compares reasonably close to the figure for repeat breeder type cattle In contrast to our results in repeat breeder cows and heifers these authors found in their study that 84% of the estrous cycles in normal animals occurred within the 18–24 day interval

Duration of heat as determined from information obtained on 26 fertile cows and heifers and 13 permanently sterile cows and heifers indicated a mean of 20 13 and 21 73 hours respectively with a variation of 8–72 hours

Many of the animals were extremely difficult to palpate with any degree of certainty therefore insufficient data were obtained on accurately timed ovulations to establish a mean Sufficient observations were made however to

9 9 mg % calcium fell on the low side of normal in both the sterile and fertile groups

Table V shows the average sodium and potassium values obtained from a total of 28 cows and heifers used in the experiment The individual hemo globin values ranged from 9 9 to 17 5% of blood but the average values fell within a narrower range The volume of the packed red cells (CV) is included for comparative purposes

Wide variations occurred in the amount of sodium in the cells and of potas sium in all three constituents These variations are attributed in part to the individuality of the cow rather than to sterility because the repeat breeder animals that eventually conceived showed almost as wide variations as the sterile cows and heifers

TABLE V

Na *and* K *Content of Blood of Fertile and Sterile Repeat breeder Cows and Heifers*

Group	No of cows	No of detn	Hb	CV
Fertile	14	33	13 1	34 0
Sterile	14	38	13 8	35 1

Group	Na			K		
	Whole blood	Plasma	Cells	Whole blood	Plasma	Cells
	mg			mg		
Fertile	280 5	337 3	168 5	55 5	20 9	122 6
Sterile	268 9	337 6	133 7	70 8	20 7	167 5

The most marked differences in the average values for the two groups appear in the amount of sodium in the cells and in the amount of potassium in the whole blood and cells There is very little difference in the sodium and potas sium content of the plasma in the two groups

The data in Table VI gives a comparison of the average values obtained from the two groups of sterile animals and values for normal cows These values are expressed as milli equivalents per liter of whole blood plasma and cells and show a decrease in the sodium content of the whole blood and cells and an increase in the potassium content of the whole blood and cells as compared to normal values

The ratio of sodium to potassium in the whole blood of the sterile cows and heifers was found to be 6 5 1 compared to 8 6 1 for normal cows and 1 4 1 compared to 2 5 1 in the cells in normal cows

When the sums of sodium and potassium are compared (Table VI) these

a varying degree of ovarian adhesion Nine out of the 14 cows and heifers which failed to conceive showed some degree of reproductive abnormality

Gestation and parturition data were available on 30 of the cows and heifers which conceived Among this group there were 21 normal and nine abnormal gestations and parturitions These figures represent a 30% incidence of gestation and parturition difficulties They include one dystocia three retained placentas one monster one uterine prolapse two abortions and one pyometritis

One cow and four heifers conceived following the administration of thyroprotein oxytocin or chorionic gonadotropin These numbers are too few to establish any conclusions however these data appear to be in agreement with Asdel s (1949) conclusions that there is very little benefit derived from hormone treatment under adequately controlled conditions

Table IV

Blood Plasma Levels of Various Vitamins and Minerals Hemoglobin Levels and Cell Volumes in Repeat breeder Cows and Heifers

Group*	1	2	3	4	1+3	2+4
Number of cows	7	18	15†	15	22	33
Number of deta	41	131	159	87	200	218
Hb %	14 5	13 5	14 5	14 0	14 0	13 7
Cell volume %	36 0	33 8	35 3	35 6	35 4	34 6
Vitamin C mg %	0 39	0 37	0 36	0 38	0 36	0 38
Vitamin E mg %	0 40	0 45	0 38	0 39	0 38	0 43
Vitamin A µg %	14 0	12 2	10 1	10 1	10 9	11 4
Carotene mg %	488 0	568 0	604 0	604 0	582 0	582 0
Ca mg %	10 1	9 8	9 8	10 1	9 9	9 9
P mg %	5 91	5 5	5 5	5 0	5 6	5 6
Mg. mg	2 52	2 74	2 77	2 89	2 7	2 8

* Group 1—control ration did not conceive Group 2—control ration did conceive Group 3—experimental ration did not conceive Group 4—experimental ration did conceive

† Includes 7 cows and heifers transferred from control to experimental ration

Biochemical determinations were obtained by standard methods for packed cell volume and hemoglobin calcium inorganic phosphorus magnesium, carotene and vitamins A C and E concentrations Blood samples were collected monthly while the cows and heifers were on the experiment Table IV summarizes the data pertaining to these determinations

No differences existed between cows and heifers on the average ration and those on the superior ration in either the infertile or fertile group Grouping the values for all cows and heifers into those which conceived and those which remained infertile failed to show any significant differences between groups regardless of the ration fed The average blood levels for all constituents were within ranges generally accepted as normal for cattle however the level of

appear to support their findings The significance of this observation is not clear however the high incidence of conception upon first service after entry on the experiment may be related to the period of rest from breeding between last service in the owners herds and first service on experiment

The data on length of the estrous period in this experiment would suggest that repeat breeder cows average 4–5 hours longer than the normal cows reported by Trimberger (1948) The method of observation and the low numbers of observations would tend to reduce the reliability of the results in the infertility experiment

The ovulation data from this experiment indicate that failure of ovulation is not an important factor in repeat breeding In those few cases in which ova were recovered upon post mortem examination there is presumptive evidence that failure of fertilization is a more important cause of repeat breeding

The incidence of gestation and parturition difficulties observed is greater than can be expected from a group of normal cows Under the conditions of this experiment however this observation is not surprising when the rigorous screening of repeat breeder cows before purchase is considered

The results of the biochemical analyses indicate that repeat breeder cows and heifers are not likely to show disturbed blood levels of vitamins A D and E and the inorganic constituents Ca P and Mg assuming that intake is sufficient to meet requirements for maintaining normal body weight and production Adreno-cortical insufficiency has been shown to cause an increased urinary excretion of sodium and a decreased excretion of potassium The repeat breeder cows and heifers in this experiment had lower whole blood and cell sodium and higher whole blood and cell potassium values than normal cows

The possibility exists that the sterility encountered in this investigation may have been caused by an endocrine disturbance The fact that adreno-cortical insufficiency causes potassium retention supports this possibility A borderline insufficiency may have been present in the cows that eventually did conceive

SUMMARY AND CONCLUSIONS

A group of 50 adult repeat breeder cows and heifers were assembled for a study of the various causes of infertility The animals were divided into control and experimental groups The basic control ration consisted of average hay and grain whereas the experimental ration consisted of high quality hay and a grain mix supplemented with vitamins and trace minerals

Approximately a 70% conception rate can be anticipated among any selected group of repeat breeder dairy cows and heifers although the protracted period of infertility would tend to make such an animal a poor economic risk

Ovulation failure was not a major cause of infertility in this group of repeat breeder cows however fertilization failure did appear to be a factor Ferti

values are almost identical in the three groups which would indicate that the total amount of these two elements in the circulating blood tends to be constant but the ratios of sodium to potassium in the cells vary widely

TABLE VI

Comparison of the Milliequivalents per Liter of Sodium and Potassium in the Blood of Normal and Repeat breeder Cattle

Group*	Na			K		
	Whole blood	Plasma	Cells	Whole blood	Plasma	Cells
	mg %			mg %		
1	121 5	146 8	75 9	14 2	5 5	30 1
2	122 0	146 7	73 3	14 2	5 3	31 4
3	116 9	146 8	58 1	18 1	5.3	42.8
Group	**Ratio Na/K**			**Sum of Na + K**		
				mg %		
1	8 6 1	26 7 1	2 5 1	135 7	152 3	106 0
2	8 6 1	27 7 1	2 3 1	136 2	152 0	104 7
3	6 5 1	27 7 1	1 4 1	135 0	152.1	100 9

* Group 1 — normal cows Group 2 — fertile repeat breeder cattle Group 3 — sterile repeat breeder cattle

DISCUSSION

The 70% over all conception rate obtained in this experiment at an average of 11 4 months would indicate that the majority of repeat breeder cows and heifers will eventually conceive upon repeated inseminations The prolonged period of infertility and the numbers of services required however, produce an economic situation for the owner that makes it difficult to justify the prolonged retention of a repeat breeder type cow in the herd.

Post mortem examination of the cows and heifers which remained sterile revealed that 78 6% had some reproductive tract abnormality which was undetected by rectal palpation Eleven of the experimental cattle which conceived were examined *post partum* Six (54 5%) had minor clinically non detectable ovarian adhesions This incidence of reproductive tract abnormalities is much higher than reported by Tanabe and Casida (1949) in cows (10 6%) and Tanabe and Almquist (1953) in heifers (13 5%) It is difficult from these data to conclusively evaluate the effect of minor ovarian adhesions on ovum migration and fertilization

Tanabe and Casida (1949) reported no significant difference between conception rate and previous numbers of services in a group of 111 cows of low fertility The conception rate and interval which we observed (Table I) would

ar to support their findings The significance of this observation is not · however the high incidence of conception upon first service after entry ıe experiment may be related to the period of rest from breeding between ervice in the owners herds and first service on experiment

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lization failure may be due in part to clinically non-detectable physical abnormalities of the reproductive tract

A high percentage of gestation and parturition difficulties can be anticipated in repeat breeder cows and heifers which eventually conceive

The addition of high carotene hay and trace minerals to the ration did not influence the blood levels of various vitamins and minerals nor was there any significant difference in the numbers of cows which conceived in either group

The repeat breeder cows and heifers in this experiment had lower blood and cell sodium and higher blood and cell potassium values than normal cows The possible relationship of adreno-cortical insufficiency to sterility has been discussed

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DISCUSSION

Tuesday July 2 1957

Morning Session

F X Gassner Presiding

OVARIAN PHYSIOLOGY

Recent Studies on the Mechanism of Ovulation in the Cow by William Hansel D T Armstrong and Kenneth McEntee Department of Animal Husbandry Cornell University Ithaca

Ovulation in the Living Albino Rat by Richard J Blandau M D University of Washington School of Medicine Department of Anatomy Seattle

A Kodachrome sound film was presented through the courtesy of Dr Blandau with running comments by Dr Phillip M Hinze Carnation Washington

The subject matter can be summarized essentially as published by Dr Blandau in his recent publication (*Fertility and Sterility* 6 5 391 1955)

Summary and Conclusions

1 A technic is described by which ovulation may be observed in the living immature rat whose ovaries have been stimulated by gonadotrophin preparations

2 The time required to complete ovulation in single follicles varies from 5 seconds to 760 seconds and depends largely upon the position of the cumulus oophorus in relation to the stigma

3 In 112 ovulations in which most of the follicular fluid escaped in advance of the ovum the mean time required to complete ovulation was 72 seconds (range 5 to 280 seconds δ 59 seconds)

4 In 51 ovulations in which the cumulus oophorus preceded the follicular fluid in passing through the ruptured stigma the mean time required was 216 seconds (range 11 to 760 seconds δ 159 seconds)

5 The time interval between the various ovulations in a single ovary varies considerably and a number of typical examples have been tabulated

6 Ovulation in the large follicles is usually explosive in nature In the follicles of intermediate size emptying is characterized by a gentle oozing out of its contents

The Effects of the Administration of Gonadotropins on the Maturation of the Egg Nucleus in Ewes by R O Berry and H P Savery Department of Animal Husbandry A & M College of Texas College Station

Relation of Nervous System to Implantation by A V Nalbandov and L E St Clair Department of Animal Science University of Illinois Urbana

Cytological Changes in the Bovine Corpus Luteum During Early Pregnancy by R C Foley and J S Greenstein Dairy and Animal Science Department University of Massachusetts Amherst.

Ovarian Function Blood Biochemistry and Reproductive Performance of Repeat Breeder Cows by Donald H McWade Clifford W Duncan and JAMES A Williams Department of Veterinary Pathology, Michigan State University East Lansing

DISCUSSION

F X GASSNER We shall open the discussion period on the presentation of this morning s papers by asking Dr Hansel to attend the lectern

H S TEAGUE I should like to ask Dr Hansel to comment on the following questions

1 What relation has nutrition to the ovulation phenomena in spontaneously ovulating species?

2 What levels of estrogen and progesterone were used at the beginning of estrus to determine effects on the time of ovulation?

WILLIAM HANSEL In reference to the first question on the effects of nutrition on ovulation there are five or six experiments now nearing completion at various places in the world all of which show rather conclusively that the initiation of first estrus and the age at which an animal first ovulates is markedly influenced by the level of nutrition In our own experiments the age of first estrus and ovulation of heifers on a low plane of nutrition is about 18 months heifers raised on a medium plane of nutrition come in heat and ovulate first at about 12 months and heifers raised on a high plane of nutrition at about 9 months In other words it is possible to speed up the process by nearly 9 months by heavier feeding On the other hand once estrous cycles have started we really have no good basis for saying that there is much difference in either regularity of estrus or in the number of ovarian abnormalities in the low medium or high fed groups These observations concern only the first 80 weeks of life it is not yet known whether differences will develop in later life as a result of different feeding levels in early life

As far as the second question is concerned the levels of progesterone used at the beginning of estrus were quite low namely 15 mg of progesterone in most cases The levels of estrogen used were also rather low I believe about 2000–4000 international units In that respect it is rather interesting that Dr Dutt (Kentucky) has conducted a similar experiment with ewes using higher levels of estradiol and has entire cycle found a significant delay in ovulation

W W BROWN Will a single dose of atropine prevent ovulation for longer than one estrus?

WILLIAM HANSEL No I think not Our data are not as good as they should be on what eventually happens to these cows after they get beyond the fifth or sixth day after ovulation was blocked because rectal palpations become difficult A few cows returned to estrus on the fifth or sixth day after the blocked ovulation ovulated normally and had normal cycles thereafter A few cows have also gone through an entire cycle without ovulating after which they came in heat and behaved normally

C F HAWKINS How do you determine when estrus begins to indicate that a heifer was slaughtered 30 minutes after that time?

WILLIAM HANSEL In heifers we checked for the beginning of estrus by using a teaser bull at 2 hour intervals In the closely timed cows we started out checking at 2 hour intervals and when it appeared as though they were getting near to estrus we checked them at 30 minute intervals

R. M COCKING I would like to pose the following questions
1 What was the dose of atropine?
2 Does this have a practical application?
3 Does the administration of oxytocin have a practical application in cows which seem to fail to conceive because of abnormally late ovulation?

WILLIAM HANSEL In regard to the first question the dose of atropine used was 40 mm/kg of body weight given subcutaneously at the beginning of estrus This is a rather large dose of atropine although it is somewhat smaller than other workers have used in laboratory animals

F X GASSNER Has this a practical application?

WILLIAM HANSEL Definitely not It is strictly an experimental tool In regard to the third question Does oxytocin have a practical role in hastening ovulation in those cases where it is delayed?" the answer is I do not know Many of you are probably aware of Van Demark s earlier report of increased conception rate in cows injected with oxytocin However I believe Dr Van Demark will agree that the question has not been adequately tested There is also some question as to how many late ovulating cows there are There are definitely some late ovulating cows but whether or not they constitute a major sterility problem in dairy cattle is unknown We are approaching this problem by attempting to use the ovulation hastening effects of progesterone and of chorionic gonadotrophin when given at the beginning of estrus to increase the conception rate in repeat breeder cows

G B MARION Were you able to determine whether or not ova resulting from delayed ovulations were normal and fertilizable?
Were resulting corpora lutea normal in appearance and function?

WILLIAM HANSEL Unfortunately we do not know the answer to either of these questions

D J REZAC Would there be an advantage to give POP intramuscularly routinely at time of artificial insemination to increase conception rate by early ovulation?

WILLIAM HANSEL Again it will be interesting to find out We simply do not know at the moment and should not jump to the conclusion that hastening ovulation will increase conception rate It may work in the opposite way in many cases

VAN DEMARK We tried injecting oxytocin along with artificial insemination by a topnotch inseminator We got no increase in conception rate when oxytocin was injected

WILLIAM HANSEL This was in contrast I believe to your earlier work where the animals were bred naturally and in which both epinephrine and oxytocin appeared to increase the conception rate

VAN DEMARK In natural mating we did get an increase in conception rate with both oxytocin injections and with epinephrine injections Likewise in a limited number of hard to settle cows with both artificial insemination and natural mating we got an increase in conception rates Using either technique on hard to settle cows the in crease in conception rate amounted to about 10-15 % With the number of animals used the difference was just barely significant

F X GASSNER Thank you Dr Van Demark Do you have anything more to say on this Dr Hansel?

WILLIAM HANSEL No more

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F HAAG At the end of your talk Dr Berry you made a remark that you found a very small number of spermatozoa present. Was this in all animals studied or was it a group which were treated with gonadotrophins?

R O BERRY I am not sure that I get your question.

F X. GASSNER Will you speak louder please

F HAAG At the end of your talk, you made the remark that you were searching for amounts of spermatozoa present in the oviduct, and it is not quite clear to me if you found a small number of spermatozoa present only in animals which received gonadotrophins or if this was generally true for others including the control animals?

R. O BERRY I was particularly searching for sperm in the animals that had been treated with gonadotrophins particularly those in the luteal phase As I recall, there was only one instance of having found any sperm in that type of animal. Whereas in the normally bred animals it was not difficult to find some sperm in the oviduct after mating

E. P REINEKE Dr Berry I believe you mentioned that when you desired to breed some of these ewes you gave an injection of stilbestrol to induce heat. I wonder whether the reproductive performance of any of those ewes was followed up and whether you obtained fertilization and conception in those cases The question that arises in my mind is whether the estrogen might increase uterine motility to the extent where retention of the ovum might be difficult or impossible

R. O BERRY Nearly all the animals in which breeding was attempted were given estrogens Not all of them came into estrus but this did not alter the situation as to the population of sperm within the tract. It did alter somewhat the cervical condition As you perhaps know the cervical mucus responds to the level of estrogens In most of our animals the cervical mucus gave what we call a negative smear but with those animals that were treated with estrogens this smear was only partly modified In other words the flame dried cervical mucus showed a slight fernlike pattern which would be typical of a normal estrous ewe I think in that way the estrogen was helpful

F X. GASSNER Does this answer your question Dr Reineke?

E P REINEKE While I am not sure whether there would be a complete answer to the question at this time I had wondered whether you did have comparisons as to actual fertility in these animals if they were carried through long enough so that you could tell whether you had actual conceptions that would be carried for any period of time

R. O BERRY The animals were all slaughtered about 60 hours after the injection so we were relying on observations as to whether or not the sperm had entered the ova or whether there was a male and female pronucleus in the egg or whether there was initiation of cleavage

E P REINEKE Thank you

F X. GASSNER Any other questions from the floor for Dr Berry Thank you Dr Berry Dr Nalbandov will you please attend the lectern

L T SAMUELS What is the relationship between Dr Berry s observation that sperm do not reach the ovum in gonadotropin induced ovulation and your observations on the importance of the uterine nerves in pregnancy?

A V NALBANDOV That is a good question We have planned some studies on the reduction in motility of the uterus after resection of hypogastric nerves We are of the opinion that the motility itself is not greatly affected by this operation that the only

F X GASSNER Anyone else from the floor? It is an important and interesting subject I should like you to participate No questions from the floor? Mr Bunding from Armour Laboratories

I BUNDING I would like to raise a question for the record as to the route of administration in Dr Van Demark s experiments

VAN DEMARK The route of injection was intravenous I have another question I would like to place to Dr Hansel Dr Hays of our laboratory has noted and I think others have also noted that frequently manual examination of anestrous cows often will precipitate estrus in such cows In more recent work by Dr Hays electrical stimulation of the reproductive cows using an electroejaculator will frequently bring these anestrous cows into estrus Dr Hansel do you interpret this to suggest that oxytocin may be involved in stimulating the estrous cycle as well as affecting ovulation as shown in your report this morning?

WILLIAM HANSEL It is possible that the hypothalamus is involved As was emphasized earlier the whole problem of the exteroceptive pathways to the hypothalamus involved and the stimulatory effect in various species is poorly understood

F X GASSNER Thank you Dr Hansel Will Dr Berry please attend the lectern for the discussion of his paper

H J HILL Did I understand that the ova released as a result of stimulation by gonadotrophins were mature cytologically but did not become fertilized as readily?

R O BERRY That is correct The eggs were just the same in appearance as those that ovulate normally but very few of them became fertilized in animals that were bred

G B MARION Do you feel that fertilization of immature eggs is impossible?

R O BERRY I would like first to have your definition of what constitutes an immature egg If by that you mean an egg in which the nucleus is in a vesicular stage or one in which there has been no division prior to ovulation I would say that it would be impossible to fertilize that type of egg

G B MARION The question was prompted by your observation that most of the normal appearing eggs which you classified as good eggs were in the second meta phase at the time of ovulation Were other ova that had not reached that stage of maturation observed after ovulation and if so was fertilization of such ova attempted?

R O BERRY No I did not observe ovulated eggs that had not matured If I had been given time to have shown you one more slide it would have indicated that about 50 / of the eggs from follicles 3 mm and over in diameter in the hormone treated ewes had started maturation. These were from follicles that did not ovulate I found none that ovulated without having produced eggs that had reached maturity

A V NALBANDOV I would like to speak on this point You are aware of the work of Noyes in which he actually found that the fertilizability of follicular eggs was as good as that of eggs that had been ovulated There is other recent work in which the same observation has been made Runnez finds that immature female mice can be ovulated with gonadotrophic hormone and these eggs too are fertilizable Of course here the question is whether these eggs are matured then by hormone treatment In the sheep data that you saw I was very much impressed by the fact that those ewes which ovulate every three or four days have eggs that are highly fertilizable at least as fertilizable as they are in the ewes that have normal 16 day cycles In these cases, the maturation process was greatly speeded up

to slaughter these heifers to get that corpus luteum picture only We have a record of heifers which were slaughtered for timed embryos which were open at all days between 16 and 33 but we certainly cannot call these normal So I do not have the answer I know we need it and we are getting various days in the cycle to contrast particularly against this 16–22 day group which were bred

J C. OSBORNE Have you made studies of the corpora lutea of laboratory animals i e mice or rats for these cell types and if so do the cell types correspond to those of the bovine?

R C FOLEY No we have not We are concentrating on cattle and we have no laboratory facilities at all for small animals in our department In relation to these cell types I omitted the review of the literature in my paper because of the time factor involved As you study the reports on the corpus luteum of the human or that of Corner in swine or others in monkeys it is easy to pick out these same cell types They use different names As you know Corner described the typical corpus luteum cell Then he added type 1 This type 1 s were our type I s and his type 2 s are our type IV's In the work of Hertig and Rock in the human his K cells are obviously type IV s I have not worked on enough rat guinea pig or rabbit corpora lutea to answer for those species

E C. REIFENSTEIN Jr Do you have information on the effects of gonadotropins either pituitary or chorionic or of gonadal hormones on the histologic appearance of the types of corpus luteum cells you have described?

R C FOLEY The Massachusetts NE 1 Regional Project which was initiated in 1954 consisted of two phases Phase two is the one that your question pertains to and we hope to start that in October In other words we have finished the timed embryo phase in pretty good shape with at least three timed embryos for each day of gestation from between day 16 to 33 We will now obtain cyclic corpora lutea from normal virgin heifers and at times we will use hormones to see whether or not we can modify the corpus luteum morphology by such treatment

G B MARION Dr Foley we are wondering whether or not you correlated the stages of corpora lutea as indicated by cell types with the previous cycling performance of these cattle prior to slaughter In other words did you have the records from the past reproductive performance of these animals so as to correlate the age of the *corpora lutea* with the animal s previous cycles thereby making it possible for you not only to classify the corpora lutea as representing a certain stage of regression but also as representing a certain number of days since the formation of each corpus luteum

R. C FOLEY We kept complete records on all the animals They were either from the University herds or from state institutions and that was of course the basis for our classification into Groups I to III When we do not have a timed embryo to check against the corpus luteum there was a question on cattle that were slaughtered after 20 days as to whether or not the animal had come in heat and gone undetected This has been a little bit of a problem on weekends at the main dairy barn We have our own herd of research animals but we also use the animals culled from the dairy herd and I was going to give you two or three interesting examples of that but there just was not enough time For example we have two 26 day corpora lutea One of the cows yielded a timed embryo and the other did not Of course the question on the one that did not is whether she had come in heat and passed unnoticed We do have a 4 day cyclic corpus luteum and in checking against that one it seemed quite obvious from the appearance of the corpus luteum that the latter 26 day corpus luteum represented a new corpus luteum and a new cycle

thing we are interrupting is the pass away from the uterus to the hypothalamus Now I know this sounds anatomically reversed but I have no explanation as to why the uterine motility is not reduced at least visually We have no measurements on this

L L LARSON What consideration will be given in the study you have proposed to the other afferent nerve fibers from the uterus namely those carried in the pelvic nerve?

A V NALBANDOV We would be very grateful for any suggestions concerning any studies that you may know of dealing with innervation of the uterus of the sheep We have not found anything that is very informative on this subject. I am sure there must be some studies that we are not aware of that would elucidate this point We have not proposed to study anything except the resection of the hypogastric nerve because this seems to do the job

F X GASSNER Any suggestions from the floor? Can anyone add to this?

L L LARSON We have done some oscilloscopic studies of the nerve supply to the genital tract both in male calves and in male and female sheep and we have found that there are afferents from the genital tract in the pelvic nerve and my thought was that if the uterus should be denervated then perhaps these should be transected as well

A V NALBANDOV I think that is a good suggestion which I will take up with Dr St Clair and see what he thinks of it Thank you very much Dr Larson

W E PETERSEN In respect to the motility of denervated uterus perhaps the observations that we made on the mammary gland may be of interest in that the denervated gland responds to the oxytocic principle as well as does the normal gland

A V NALBANDOV I think that is a very interesting observation

R O BERRY Have you tried putting artificial embryos in animals other than the ewe?

A V NALBANDOV Yes this work was originally started on guinea pigs and is now being continued on that species by Dr W W Moore (now at the University of Indiana) while he was associated with me in the sheep work at Illinois He told me recently that the effects he gets are the same as those observed in sheep There is also a paper by Velardo and Hisaw in which it was shown that the size of placentoma in rats is directly proportional to the length of the period of pseudo pregnancy The smaller the placentoma in the uterus the shorter the pseudo pregnancy The larger and the more placentoma there are the longer pseudo pregnancy This seems to fit into the consideration that we are talking about

F X GASSNER Dr Foley please attend the lectern for discussion of your paper

M A BROWN Will you give relative percentages of the various types of luteal cells in mid-cycle nonpregnant corpora lutea in contrast to what is seen in those of early pregnancy?

R C FOLEY I was prepared for that question but I doubt that I will be able to answer it Our first objective was to get timed embryos Incidentally we do serial sections on all of these embryos to get a standard of development We concentrated on the 16 17 and 18 day pregnancies since we had no passed over estrous periods to guide us However we are now just about finishing the embryo series and are up in the 30–33 day group We are also just beginning the analyses on the corpora lutea from normal cycling heifers In other words, it seemed like a lot of wastage in some ways

R. C. Foley That is one we have not worked out as yet As I told you we have nineteen of them and the impression that I have from scanning them is that the cell types III IV, and V in the abnormals predominate but whether or not there is a difference between an abnormal corpus luteum with and without a cavity we do not know yet. I am under the impression that there is not a marked difference

F X. Gassner Dr McWade s paper is now open for discussion

C Branton Was thyroidal activity (PBI) determined on the cows? If not, why not, since you indicated that certain cows received thyroprotein therapy?

D H McWade There was only one instance when we attempted to determine the protein bound iodine in these cows Thyroprotein additive was arbitrarily de cided upon in the original experiment We did not continue with the protein bound iodine determinations because of lack of facilities

V W Zuercher Do you know of any authentic cases of pregnancy in cows that were inseminated after the appearance of bloody discharge?

D H McWade No

Pierre Lieux You mention using thyroprotein when treating some repeat breeders Could you state the dose and method of administration?

D H McWade Thyroprotein was given orally with the grain at approximately 10 g daily over a period of 30 days

E. P Reineke Were there any indications in the structure or microscopic anatomy of the adrenal cortex that could be correlated with the altered sodium–potassium ratio found in the sterile cows?

D H McWade We are in the process of evaluating the tissue sections taken during this past experiment. So far there are no indications of insufficiency related to the sodium–potassium levels

G B Marion I would like to comment on some of the work we have going which is probably very similar to Dr Foley s work We have followed the cows to slaughter that have been removed from the Kansas State dairy herd either to attempt recovery of embryos or for fertility reasons At the present time our work is apparently somewhat in continuation of Dr Foley s because our embryo and fetal series range from approximately 30 to 240 days However we have a great number of cows that were sent to slaughter because of lack of normal reproductive performance and from those animals we have recovered a great number of corpora lutea from ovaries representing various stages of the estrous cycle We have been able by studying the anatomical histological and histochemical pictures obtained from these corpora lutea and correlating these findings with complete reproductive records maintained on these animals prior to slaughter to determine the exact age of each corpus luteum in an ovary We can thereby trace each corpus luteum back to the particular cycle it happens to represent. I do not want to challenge any of Dr Foley s grand material which was so well presented However we would like to present a little different aspect of this thing We feel that we have enough information to state that the cells which he classified as type I are probably of theca interna origin The cells of considerable larger size probably represent the granulosa cells and that the type 3 cells are actually the secretory cells that are responsible for progesterone secretion We have a number of very young corpora lutea representing 1 2 3 4 days and older following ovulation on which our conclusions are based

R C Foley May I ask a question On what basis did you assign the secretory function to the type 3 cells?

G B Marion Our determinations were based mainly on histochemical evaluations The presence of fat in the cells was one criterion Furthermore the fact that some of the large cells were more granular and deeper staining as were your number IV cells might indicate that they have gone beyond the secretory phase In other words this would be similar to some of the work done with the pituitary gland where degranulation indicates the actual function of the cell rather than the presence of granules indicating actively secreting cells We are not certain of some of these theories however working with both frozen sections and the normal histochemical techniques and correlating the findings of both techniques our results seem to be consistent with these thoughts

R C Foley I would like to ask just one more question What lipoid stains did you employ?

G B Marion We used both Sudan black and Sudan IV as well as hemotoxylin and counter stain and found the lipid material to be rather consistent

R C Foley We are using Sudan black and getting particularly good results with Oil red O However we are still confused on the significance of both of these stains I purposely avoided the lipid story because we just do not know what the answer is yet A lot of people have been working with these fat stains in other species and they do not seem to come out with very positive answers and I am withholding judgment until we can get a better understanding of our own results

G B Marion I would like to say amen to that last statement We have considerable material which is somewhat confusing as well but a majority of our material is consistent and so possibly adds another theory to the action of corpora luteal cells where they originate and what function they perform

William Hansel What are predominating cell types in corpora lutea with large central cysts?

III STEROID PHYSIOLOGY AND THERAPY

BIOSYNTHESIS OF STEROID HORMONES

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TEN years ago this title would have had relatively little meaning At that time nothing was known of the biosynthesis of the steroid hormones except the observations of Sayers *et al* (1944) that injections of ACTH led to a fall in the cholesterol and ascorbic acid of the adrenal and that this was associated with evidence of increased adrenal hormone production Today we can present a general outline of the synthesis of all of the steroid hormones which while not necessarily perfect in all of the steps indicated is probably very close to the truth

This will be a general review embodying certain concepts First there seems to be a common path of synthesis Nature has used a general route for the formation of both the reproductive and the adrenal hormones Second the differences both between species and between those organs which form the steroid hormones are quantitative and not qualitative This explains why abnormalities in the development of these organs can lead to any type of steroid hormonal excess Third hormonal control is achieved in the organism by the rapid formation and breakdown of these hormones The products formed by metabolism are not those which are used in biosynthesis thus there is little or no feed back and the levels in the circulation are proportionate to the stimulus for release Fourth from the stage of steroid ring formation on only two coenzymes seem to play important roles diphosphopyridine nucleotide and reduced triphosphopyridine nucleotide The latter is particularly vital in a number of the reactions which take place It is important to draw attention to this because here we may find the explanation of the action of the trophic hormones

Our knowledge of steroid metabolism has developed rapidly in the last 10 years because of two major technical advances first the development of chromatography and particularly paper chromatography which has made possible the separation of very small amounts of material from large amounts of very similar compounds and second the availability of radioactive carbon which could be incorporated into organic compounds The latter has been important not only because it makes possible the identification of metabolic products in very small amounts as such but also because the trapping tech

examining cholesterol synthesis in the liver (Bloch 1956) Apparently the acetate is first involved in the formation of acetoacetic acid The acetoacetic acid in turn reacts with further acetate to form 6 carbon acids which have a branched chain similar to the structure found in the isoprene unit of rubber The exact acids involved here are under dispute because these can only be identified by the trapping technique or by addition of the acid and determination of incorporation but it does seem that these acids are finally combined to form a 15-carbon acid farnesinic acid (Dituri *et al* 1956) and that two of these units unite to form a hydrocarbon This of course is a reductive series of reactions

The hydrocarbon squalene was originally identified in the livers of sharks as its name implies It had not been identified in any other source until traces of it were found in human skin Langdon and Bloch (1952) using the trapping technique found that when acetate was fed and squalene was also supplied to the animal radioactive squalene could be isolated from the liver Using the radioactive squalene so obtained they showed that cholesterol could be formed from this material

Squalene can be so folded that it will by forming cross bonds at points involving a double bond yield another substance which has the four rings characteristic of the steroid hormones and cholesterol (Woodward and Bloch 1953 (FIG 1)) This is lanosterol the sterol from wool fat which again had not

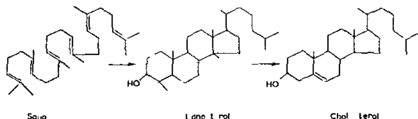


FIG 1 Biosynthesis of cholesterol from squalene

been recognized as an important general metabolite By using the trapping technique however Loud (1956) and Clayton and Bloch (1956a b) have been able to show that both squalene and lanosterol are very rapidly turned over in the animal and that cholesterol is rapidly formed from them this accumulates because its further metabolism is much slower than that of the previous two compounds From the point of ring formation on triphosphopyridine nucleotide in its reduced form and atmospheric oxygen were found to be necessary This system plays an important part in many of the subsequent steps in the formation of the steroid hormones

Not only does the synthesis of cholesterol from acetate occur in the liver but it happens in the adrenal (Srere *et al* 1948) the testis (Brady 1951) and

nique can be used That is a large amount of non radioactive intermediate can be introduced into a metabolic system and any of this compound formed from a radioactive precursor will then exchange with it The major portion undergoing further metabolism will then be non radioactive Thus the radio activity is trapped at this point and can be identified by subsequent isolation where it might not be possible to find the amounts that exist as such, even as a radioactive compound

The first break through in the solution of the problem of biosynthesis was the work done at the Worcester Foundation for Experimental Biology where Hechter *et al* (1949) perfused adrenal glands and showed that the introduction of radioactive acetate of radioactive cholesterol led to the formation of radio active adrenal hormones They also demonstrated that if ACTH were added this conversion could be increased in fact the conversion without the presence of ACTH was very small Two important factors were thus established that acetate and cholesterol could both be precursors and that the trophic hor mones could act directly and promptly in this synthetic conversion

Saffran *et al* (1952) and others demonstrated that ACTH could have this action when just slices were introduced with radioactive acetate However when homogenates of the glands were used it was found that ACTH did not increase the conversion of acetate or cholesterol to these steroid hormones There have been reports that the trophic hormone could have an effect in homogenates but these experiments as a whole have not been duplicated, and at the present time it would seen that the action of the trophic hormone can be demonstrated clearly only in the presence of intact cells Moreover it was dis covered that when an excess of the nucleotides was added the production of steroid hormones by the homogenates was as large as that which was achieved with the slices incubated with added ACTH

These observations were made with adrenals but in 1951 Brady demon strated that slices of testes from a wide number of animals would also convert acetate to the androgenic hormone testosterone He showed that if he added chorionic gonadotropin to these slices he achieved greater incorporation of the acetate The Utah group (Samuels and Helmreich 1952) found that acetate was converted to testosterone and androstenedione by testis homogenates but was not able to find any effects of the chorionic gonadotropin *in vitro* If however the chorionic gonadotropins were previously injected the ability of the animal to cause conversion was increased More than that such testes had greater specific hormonal and enzyme effects (Samuels and Helmreich 1956) so there was a difference between the *in vitro* effect which could be immediately achieved and which could only be demonstrated in slices and the effects which could be obtained with homogenates where the gonadotropin had been allowed to act in the animal previously

The series of reactions involved in steroid biosynthesis begins with the syn thesis of cholesterol from acetate Bloch *et al* have studied this problem by

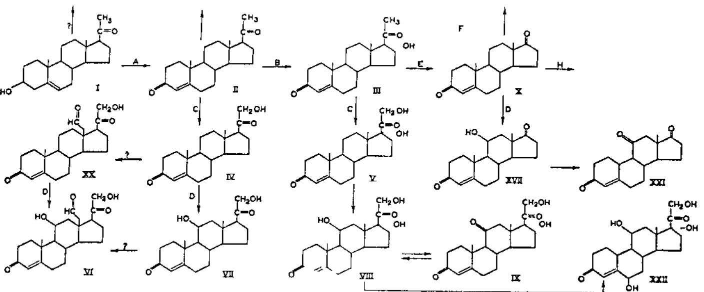


FIG 3 Biosynthesis of adrenal steroids from 5 pregnen 3β ol 20-one

in all those places where steroid hormones are formed (Popjak and Tietz, 1953 Heard *et al*, 1954)

The next steps in the conversion of cholesterol to steroid hormones have been worked out by Staple *et al* (1956) The largest steroid hormone molecule which we know is the adrenal hormone cortisol, which has 21 carbons This 21 carbon structure now appears to be formed by hydroxylation of cholesterol at carbon 20 then splitting at this point with the formation of isocaproic acid and a compound which had been isolated from the testis, the adrenals, and possibly the corpus luteum 5 pregnen 3β ol 20 one (FIG 2) These steps again require the presence of reduced triphosphopyridine nucleotide and oxygen

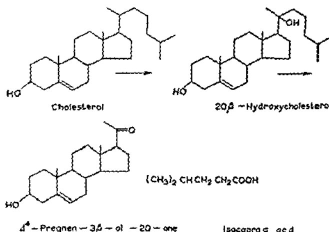


FIG 2 Formation of 5 pregnen 3β ol 20 one from cholesterol

Our own group found that when 5 pregnen 3β ol 20-one was incubated with adrenal testicular placental or ovarian tissue progesterone was obtained It did not seem to make any difference which tissue was used progesterone was always the product when diphosphopyridine nucleotide (DPN) alone was cofactor (Samuels *et al* 1951) For the next steps some source of reduced triphosphopyridine nucleotide (TPNH) had to be used, and oxygen was essential These cofactors were always required when hydroxyl groups were introduced A series of enzymes each using these cofactors but a different substrate has been shown to lead to the secretory products of the adrenal (FIG 3)

One of the important hormones of the adrenal, other than cortisol and corticosterone is the substance aldosterone Kahnt *et al* (1955) were able to show that progesterone or desoxycorticosterone could be converted to aldosterone by incubation with adrenal tissue and more recently the discoverers of aldosterone Tait and Simpson (Ayres *et al* 1957) have shown that if they use the outer part of the adrenal cortex they can get rapid formation of this compound from either desoxycorticosterone or corticosterone

requires triphosphopyridine nucleotide and oxygen as in the simple introduction of a hydroxyl group Further it was found that 17α hydroxyprogesterone appears to be the best substrate while if a second hydroxyl group is introduced, as in 17 hydroxydesoxycorticosterone then the material will not go directly to the carbon 19 androgens (Lynn and Brown 1956) There appears to be a preferential sequence

More recently it has been shown that testosterone can be converted to estradiol by human ovaries (Baggett *et al* 1956) and in the pregnant mare (Heard *et al* 1955) Also Meyer (1955a b) demonstrated that a number of tissues such as ovary adrenal and placenta will oxidize androstenedione to the 19 hydroxyandrostenedione and it in turn will be converted into the estrogen estrone These sequences have not been worked out so thoroughly as the others thus far but again starting with a compound the origin of which can be traced the estrogens are obtained So from a common precursor pregnenolong in the presence of enzymes of these various tissues one can form all of the steroid hormones with which we are acquainted

Now the question arises Do all of these reactions occur in all of the tissues? *We don t know Obviously there are marked differences but we have evidence* that these are not absolute differences They are quantitative and not qualitative Slaunwhite and Samuels (1956) found evidence for the presence of compound A of Kendall (dehydrocorticosterone) in incubations of testis slices and material is always obtained which has 21 carbons and is quite polar In testicular tumors Samuels (1957) has been able to demonstrate formation of desoxycorticosterone Also Zander (1957) has found that the corpus luteum not only contains progesterone which of course is its major hormonal component but also 17 hydroxyprogesterone and androstenedione It may be that the androstenedione is an intermediate in the formation of estrogens by the human corpus luteum

So much then for the series of reactions which lead to the steroid hormones and the evidence that the differences between organs are quantitative rather than qualitative Short consideration will be given to the present status of the mechanism of action of ACTH and HCG (chorionic gonadotropin) If a single intravenous injection of chorionic gonadotropin is given to a dog and the blood from the testis is collected thereafter within the first half hour there is a marked increase in the output of both androstenedione and testosterone the ratio between the two remaining relatively constant (Brinck Johnsen 1957) This as pointed out earlier could not be demonstrated in homogenates and when slices were compared with homogenates the production of homogenates reinforced with the proper cofactors approaches that which can be obtained by the slices stimulated by the tropic hormones Recently Haynes and Berthet (1957) have introduced evidence that ACTH increases the activity of an enzyme phosphorylase Phosphorylase has long been known as an enzyme in carbohydrate metabolism but there are probably different phos

Slaunwhite and Samuels (1956) demonstrated that when progesterone was incubated with testis slices one could obtain androstenedione which is a good androgen and which West *et al* (1952) showed was secreted into the blood by the testis along with testosterone (FIG 4) When progesterone with radioactive carbon 21 was incubated with testis slices 50% of the radioactivity was soluble in ethyl acetate 42% remained in aqueous solution and a small

FIG 4 Probable biosynthesis of androgens and estrogens

amount (about 8%) still remained attached to the protein precipitate Very little was found in the aqueous solution when ring labeled progesterone was used *so apparently something happened which split off carbon 21 and made it* water soluble while carbon 4 was not affected These workers went further and showed that when carbon 4 was marked radioactive androstenedione and testosterone could be found after incubation of progesterone while if carbon 21 were marked no activity was found in these compounds They showed that the radioactive compound formed when carbon 21 was split off was apparently a volatile acid Since that time the evidence seems to point to acetic acid as the product which is split off

Martin (1955) found that the sequence actually was an oxidation of 17 hydroxyprogesterone to androstenedione followed by reduction to testosterone and not a hydrolytic reaction yielding acetic acid and testosterone followed by oxidation to androstenedione Thus side chain splitting reaction

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phorylases in different tissues Such words have been used as a collective term and we sometimes forget that enzymes catalyzing the same reaction can differ between different tissues This is seemingly very important in hormonal action The phosphorylase in the adrenal apparently is activated by ACTH leading to a greater conversion of glycogen to glucose 6 phosphate, and this in turn can supply energy to the systems which produce triphosphopyridine nucleotide in reduced form Triphosphopyridine nucleotide unlike diphos phopyridine nucleotide is only present in tissues in very small concentration It is higher in the adrenal and the testis than in most tissues but it is still low and can be the limiting factor since it enters into so many of the reactions involved in the sequential synthesis It may be therefore that the action of these tropic hormones is not directly on some specific enzyme in the biosyn thetic sequence but to supply this limiting cofactor so that the ultimate out come is to increase the whole sequence of reactions involved in synthesis This remains to be proved but the evidence is sufficient to indicate that we should look in this direction

Thus we see that there is a sequence of biosynthetic steps which can be traced all the way from acetate through cholesterol and pregnenolone to each of the steroid hormones While enzymes catalyzing certain steps predominate in specific tissues there is evidence that the others are not necessarily com pletely absent and abnormalities which lead to production of hormones not usually associated with a tissue would not require mutational change Lastly the control of production by the tropic hormones may be not through action on an enzyme catalyzing a specific step in steroid biosynthesis but through influence on the production of a cofactor present in limited amounts

COMPOUNDS IN FIGURES 3 AND 4

I Δ^5 pregnen 3β ol 20-one (Pregneno lone)
II Progesterone
III 17α hydroxyprogesterone
IV Deoxycorticosterone
V 17α hydroxydeoxycorticosterone (Reichstein s Compound S)
VI Aldosterone
VII Corticosterone
VIII 17α hydroxycorticosterone (Ken dall s Compound F) (Cortisol)
IX Cortisone (Kendall s Compound E)
X Δ^4 androstene 3 17 dione (Androstenedione)
XI Testosterone
XII Estradiol
XIII Estrone
XIV Estriol
XV 17α hydroxypregnenolone
XVI Dehydroisoandrosterone
XVII 11β hydroxyandrostenedione
XVIII 6β hydroxyprogesterone
XIX 19 hydroxy Δ^4 androstene 3 17 dione
XX 18 aldo 11 deoxycorticosterone
XXI Adrenosterone (Δ^4 androstene 3 11 17 trione)
XXII 6β Hydroxycortisol

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THE MAINTENANCE OF HUMAN PREGNANCY WITH PROGESTATIONAL COMPOUNDS*

EDWARD C REIFENSTEIN Jr M D

ENDOGENOUSLY PRODUCED hormones with progestational activity have im portant physiologic functions in the maintenance of the normal course of gestation in animals and in man Consequently in both of these species *therapeutically administered compounds with potent progestational activity* must have important roles in the management of those gestations which fol low an abnormal course because the endogenous support with progestational hormones is not adequate

This concept has not been accepted universally because the favorable in fluence upon pregnancy complications of treatment with the naturally occurring hormone progesterone has not been easy to demonstrate The essential problem has been to supply therapeutically the quantities of proges tational activity which apparently are required for the successful maintenance of pregnancy The difficulties with progesterone preparations have resulted from three factors (1) the steroid has a very short duration of action (2) it has a low solubility in oil and weak activity when administered by routes other than by injection and (3) the existing oily solutions or aqueous suspen sions of this compound are poorly tolerated following injection and cause manifestations of local irritation

The purposes of this presentation are (1) to give a report of preliminary observations on the effectiveness of a new long acting progestational agent 17 α hydroxyprogesterone caproate† in the maintenance of human preg nancy and (2) to stimulate further evaluation of this steroid ester in the treat ment of pregnancy complications in animals The evidence indicates that in man hydroxyprogesterone caproate has none of the undesirable features which have hampered the full clinical application of free progesterone

THE DISCOVERY OF HYDROXYPROGESTERONE CAPROATE†

In 1954 Junkmann prepared thirty eight derivatives and esters of proges terone and determined their biologic activity by the Clauberg test He dis

From The Medical Division The Squibb Institute for Medical Research E R Squibb and Sons Division Olin Mathieson Chemical Corporation New York, and the Department of Medicine New York Medical College Flower and Fifth Avenue Hospitals New York N Y

† Delalutin, E R Squibb & Sons New York

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progestational compound) The regimens used to supply the estrogenic action are given in Table I Comparable dosage levels of the two progestational agents were administered in the trials that are compared the regimens employed are listed in Table II

TABLE I

Regimen Employed for Priming and Concomitant Treatment with Estrogens

Type of estrogen	Compound	Amount	Number of doses	Interval between doses	Time of giving estrogen to time of giving last dose of progestational agent	
					First dose estrogen	Last dose estrogen
Short acting (21 trials)	Estradiol benzoate	5 mg	4 injections	3–4 days	14–17 days before	simultaneously
Long acting (23 trials)	Estradiol valerate*	5–20 mg	2 injections	7–14 days	7–17 days before	simultaneously
Continuous (8 trials)	(*a*) Diethyl stilbestrol	1 mg	dail p o	one day	14 days before	continued daily after progestational agent to desquamation.
	(*b*) Endogenous	†	see below	see below	see below	see below

* Delestrogen E R Squibb & Sons New York

† Endogenous estrogen adequate for full secretory endometrium at desquamation proved by biopsy in a preliminary trial with progestational agent

The results obtained by this procedure with *free progesterone* are given graphically in FIG 1 The duration of action of this steroid averaged 4 0 days with short acting estrogen 6 1 days with long acting estrogen 4 5 days with continuous estrogen and 5 1 days with all types of estrogenic activity combined The results with Delalutin are shown graphically in FIG 2 The duration

covered that esterification with caproic acid of free 17 α hydroxyprogesterone (a compound with minimal activity as a progestogen (Junkmann 1954 Kessler and Borman 1957)) to produce 17 α hydroxyprogesterone 17 *n* caproate resulted in a steroid ester with considerably prolonged duration and greatly increased degree of progestational activity Furthermore the ester had sufficient solubility in oily vehicles (sesame oil with benzyl benzoate castor oil with ethyl lactate and castor oil with benzyl benzoate) for use in human therapy The progestational action of hydroxyprogesterone caproate in animals has been confirmed by Kessler and Borman (1957) The author elsewhere (1957) has summarized some other published reports on the physiologic properties of this compound in animals and in man and on the initial clinical experience in gynecologic and obstetric disorders The caproate ester is available under the brand name Delalutin which for convenience will be employed hereafter in this presentation

A COMPARISON OF CERTAIN PROPERTIES OF HYDROXYPROGESTERONE CAPROATE (DELALUTIN) WITH THOSE OF FREE PROGESTERONE

The investigations in man (Reifenstein 1957) permit a comparison of certain properties of Delalutin with those of free progesterone Three points will be considered briefly (1) the duration of action (2) the solubility in oil and (3) the freedom of patients from reaction following intramuscular injection

1 *Duration of Action* An index of the duration of action of the two progestational compounds was obtained by comparing the intervals between the last injected dose of the agent and the onset of desquamation of a mature secretory endometrium The analysis is based on the data of four groups of investigators (Boschann 1954 1955a 1955b Davis and Wied 1955 Cohen *et al*, 1956) which consist of 52 trials in 14 patients (4 castrates with an intact uterus whose ages ranged from 32 to 42 years and 10 patients with secondary amenorrhea whose ages ranged from 21 to 26 years) Estrogenic hormones were administered to these women for two objectives (1) to prime the uterus and induce endometrial proliferation and (2) to supply estrogenic activity concomitantly while the progestational agents were exerting their influence Three types of estrogenic action were evaluated (1) short acting estrogenic activity (estradiol benzoate by injection) (2) long acting estrogenic activity (estradiol valerate* by injection) and (3) continuous estrogenic activity (diethylstilbestrol by mouth daily for 2 weeks before the administration of the progestational substance and continued thereafter each day until desquamation or endogenous estrongenic activity proven by biopsy to be adequate for a full secretory endometrium at desquamation in a preliminary trial with the

* Delestrogen E R. Squibb & Sons New York.

long acting or continuous estrogenic activity was available The results are presented graphically in Part 1 of FIG 3 With adequate estrogen the average

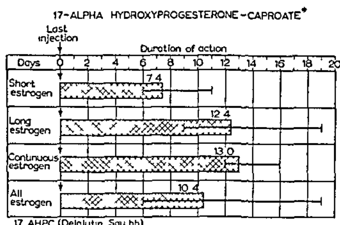
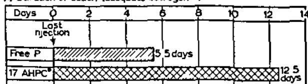
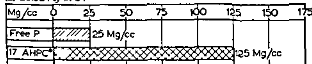
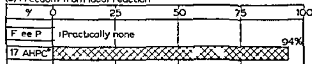


FIG 2 The duration of action of 17 α hydroxy progesterone-caproate (Delalutin)
For discussion see text The regimen employed with estrogen for priming and concomitant administration with the progestational agent is given in Table I The regimen employed with the progestational agent is given in Table II The range of response in the trials is shown by the black bar (*Prepared from the data compiled by Reifenstein 1957*)

COMPARISON OF 17 ALPHA-HYDROXYPROGESTERONE-CAPROATE* AND FREE PROGESTERONE







17 Alpha Hydroxyprogesterone Caproate; 17-AHPC (Delalutin Squ bb)
Delestrogen or cont nuous estrogen

FIG 3 Comparison of three properties of 17 α hydroxyprogesterone-caproate (Delalutin) and of free progesterone
For discussion see text (Prepared from the data compiled by Reifenstein 1957)

TABLE II

Regimen Employed for Treatment with Progestational Agent

Type of progestational agent	Compound	Amount	Number of doses	Interval between doses	Number of trials
Short acting (15 trials)	Progesterone	75–100 mg	4–5	one day	5 trials
		150–350 mg	1	—	10 trials
Long acting (37 trials)	17 AHPC*	65–125 mg	2	6 days	11 trials
		250–500 mg	1	—	26 trials

* 17 α hydroxyprogesterone-caproate (Delalutin E R Squibb & Sons New York)

of action of the steroid ester averaged 7 4 days with short acting estrogen 12 4 days with long acting estrogen 13 0 days with continuous estrogen and 10 4 days with all types of estrogenic activity combined

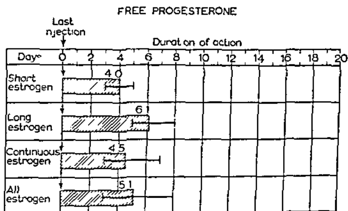


FIG 1 The duration of action of free progesterone
For discussion see text The regimen employed with estrogen for priming and concomitant administration with the progestational agent is given in Table I The regimen employed with the progestational agent is given in Table II The range of response in the trials is shown by the black bar
(Prepared from the data compiled by Reifenstein 1957)

It is apparent from FIGS 1 and 2 that insufficient estrogenic activity was available during the action of both progestational agents when short acting estrogen was employed since under these conditions the shortest duration was obtained with both compounds Therefore to obtain an index of the duration of the effect of these two agents when *adequate estrogenic activity* was present, averages were calculated by combining the trials in which either

TABLE III

The Effectiveness of Free Progesterone in Habitual Abortion
(All patients with three or more consecutive abortions)

Data of Davis and Plotz (1957)

	Number of patients	Number of pregnancies	Number living babies	Salvage rate
Past record	90	289	40	13 8
Progesterone Rx pregnancy	90	90	63	70 0

a fetal salvage rate of 70 0% Thus adequate and sustained progestational therapy resulted in over three times as many successful pregnancies with live babies in these patients However the authors stressed the inconvenience and impracticality of months of daily injections of free progesterone

THE EFFECTIVENESS OF DELALUTIN IN HABITUAL ABORTION

Because of the advantageous features of Delalutin we began as soon as this compound became available to collect reports of cases of recurrent abortion treated with it The observations on 82 cases formed the basis for this prelimi nary communication The reports were made available for this compilation through the kind cooperation of 23 different investigators who administered the caproate ester to their patients and recorded the responses to it The series includes only patients who have had at least three previous abortions all (except two*) had classical habitual abortion The series is restricted to patients who did not receive estrogen in addition to Delalutin no restriction was made on the basis of the use of other non hormonal medication Each physician who has employed the caproate ester has devised his own dosage schedule and regimen of administration Therefore the cases in this analysis have been limited to (1) those that have received the steroid ester by injection in a dosage of at least 125 mg every 10 days (2) those that were given at least three in jections of the hormone preparation and (3) those that were started on treatment not later than the 12th week The average dosage for the entire series was approximately 250 to 375 mg once a week throughout the treatment period therapy usually was started by the 5th week and continued to the 30th week of gestation However there was considerable variation (1) the dosage ranged from 125 to 875 mg per week (2) the interval between injections ranged from once in 10 days to three times a week (3) the maintenance program in cluded cases on constant dosage increasing doses decreasing doses and in

* It is our intention to include only cases with *three consecutive* abortions After the statistics and illustrations for this preliminary report were prepared we discovered that the three abortions in two of the women were not consecutive These cases will be eliminated in the final series

duration of action of free progesterone was 5 5 days while that of Delalutin was 12 5 days

2 *Solubility in Oil* Other clinically important properties of the two compounds also are compared in FIG 3 Free progesterone can be dissolved in oil to form a true solution only to the extent of 25 mg/ml (cm³) whereas Delalutin forms a true solution in oil in an amount of 125 mg/ml (FIG 3 Part 2)

3 *Freedom from Reactions* Another important difference between the two progestational agents is the incidence of local reactions following intramuscular injection (FIG 3 Part 3) Practically none of the patients in this series (or of other individuals who were given free progesterone in solution or in oily or aqueous suspensions) was free from some local manifestation of irritation (such as pain tenderness or induration) In contrast 94% of a series of over 500 patients who received a single injection of Delalutin in doses ranging from 125 to 500 mg was completely free from local reactions

4 *Advantages of Delalutin over Free Progesterone* The comparison of the two progestational agents indicates that Delalutin has at least twice the duration of action five times as much active material per unit volume and a very markedly greater freedom from local reaction after injection than free progesterone These advantageous features immediately suggested the potential usefulness of Delalutin in patients with habitual abortion

THE EFFECTIVENESS OF FREE PROGESTERONE IN HABITUAL ABORTION

Recently Davis and Plotz (1957) in an important study demonstrated the effectiveness of adequate and sustained progestational therapy with free progesterone in habitual abortion This investigation has been in progress for the past 5 years at the Chicago Lying in Hospital Only women who had had at least three consecutive abortions were included in the series in other words these patients had classical habitual abortion Treatment with free progesterone was started immediately after the first missed menstrual period and continued either (1) until the patient felt life at about the 16th to 18th week of gestation or (2) if the pattern of previous abortions indicated difficulty at mid pregnancy until the baby reached a safe period of viability at about the 34th week The free progesterone was administered by injection in a dosage of 100 mg daily for 4–5 days each week This schedule of treatment was difficult to administer because it required frequent and often long trips to the hospital and because the injections caused considerable discomfort from the accumulated oil in the muscles

In spite of these unfavorable features the therapeutic program was completed by 90 patients (Table III) Prior to the gestation treated with free progesterone the 90 women had had 289 pregnancies with 40 living babies a fetal salvage rate of 13 8% During the 90 pregnancies in which free progesterone was administered as previously described, there were 63 living babies

Although four of these patients were started on the steroid medication before the 5th week of gestation relatively small amounts of the compound were employed In contrast in the other three women who aborted with missed abortion the pregnancies terminated in spite of moderate to large doses of the agent It is of interest also that approximately one quarter (12 cases) of the 53 women who delivered live babies following treatment with Delalutin had *premature labor* (at 30 to 36 weeks) Two-thirds of these patients were started on therapy during the 6th or 7th week of gestation One-half had been on moderate or small doses which were discontinued for ten or more weeks before the onset of the premature labor In contrast three women developed spontaneous labor prematurely while still receiving the caproate ester The difference in the therapeutic regimens employed by the collaborating investigators and the occurrence of missed abortion and of premature labor in some of the cases support the interpretation that not all of the women in the series were given optimal amounts and/or administration schedules of Delalutin

COMPARISON OF THE EFFECTIVENESS OF DELALUTIN WITH THAT OF FREE PROGESTERONE IN HABITUAL ABORTION

The effectiveness of Delalutin therapy in increasing the fetal salvage rate of the 82 women with habitual abortion in this series is compared with that of free progesterone in improving the fetal salvage rate of the 90 women with habitual abortion reported by Davis and Plotz (1957) in FIG 4 In spite of the

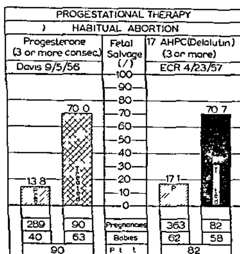


FIG 4 Comparison of the effectiveness of 17-α hydroxyprogesterone-caproate (Delalutin) with that of free progesterone in habitual abortion

For discussion see text The data for this figure are given in Tables III and IV Prev = previous pregnancies not treated with a progestational agent Treated = pregnancies treated with one of the progestational agents 17 AHPC = 17-α hydroxyprogesterone caproate (Delalutin Squibb)

creasing then decreasing dosage (4) the time of starting therapy ranged from the 2nd to the 12th week of gestation and the time of voluntarily terminating the therapy ranged from the 15th to the 38th week Because of these variations, some of the patients included in the series may not have received adequate amounts of the progestational agent

The results on the 82 patients are given in Table IV Prior to the gestation that was treated with Delalutin the 82 women had had 363 pregnancies with

TABLE IV

The Effectiveness of 17 α Hydroxyprogesterone caproate (Delalutin) in Habitual Abortion*

Number of cases	82
Previous Record	
No of pregnancies	363
No of babies	62
No of abortions	301
Salvage rate	17 1%
17 AHPC Therapy*	
No of pregnancies	82
No of babies	53
No viable cases	5
Total babies and viable	58
No of abortions	24
Salvage rate	70 7 %
Missed abortions	7†
Other abortions	17
Total abortions	24
Premature labor	12‡
Delivered no complications	40

17 AHPC = 17-α hydroxyprogesterone-caproate (Delalutin E R Squibb & Sons New York)
† Approximately 1/3 of total abortions
‡ Approximately 1/4 of total deliveries

62 living babies a fetal salvage rate of 17 1% During the 82 pregnancies in which Delalutin was administered as previously described there were 58 living and viable babies (53 delivered and 5 viable (over 28 weeks of gestation)) a fetal salvage rate of 70 7% Thus adequate and sustained progestational therapy *with Delalutin* resulted in over three times as many successful preg nancies with live babies in these patients The investigators without exception were very favorably impressed with the convenience and practicality of using 17 α hydroxyprogesterone caproate in the treatment of abortion and with the almost complete freedom from local reactions to the injections in spite of the administration of large amounts (up to 5 cm³ as a single dose)

It will be noted in Table IV that approximately one third (7 cases) of the 24 patients who aborted in spite of Delalutin therapy had a *missed abortion*

Although four of these patients were started on the steroid medication before the 5th week of gestation relatively small amounts of the compound were employed In contrast in the other three women who aborted with missed abortion the pregnancies terminated in spite of moderate to large doses of the agent It is of interest also that approximately one quarter (12 cases) of the 53 women who delivered live babies following treatment with Delalutin had *premature labor (at 30 to 36 weeks) Two thirds of these patients were* started on therapy during the 6th or 7th week of gestation One half had been on moderate or small doses which were discontinued for ten or more weeks before the onset of the premature labor In contrast three women developed spontaneous labor prematurely while still receiving the caproate ester The difference in the therapeutic regimens employed by the collaborating investigators and the occurrence of missed abortion and of premature labor in some of the cases support the interpretation that not all of the women in the series were given optimal amounts and/or administration schedules of Delalutin

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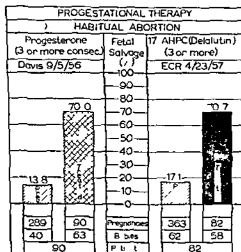


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fact that the patients treated with Delalutin by twenty three different investigators did not receive uniformly as much or as sustained progestational therapy as those treated with free progesterone by Davis and Plotz, essentially the same fetal salvage was achieved with both compounds With Delalutin the fetal salvage rate rose from 17 1 to 70 7 and with free progesterone from 13 8 to 70 0 The important differences between the results with the two progestational agents are (1) *the improvement in the fetal salvage rate was accomplished with an average of one injection a week with Delalutin* in contrast to an average of five injections a week with free progesterone and (2) *the patients receiving injections of the caproate ester were practically free from local reactions* whereas almost all of those given the free compound had local discomfort with pain or tenderness at the sites of injection

DISCUSSION

From the data presented, failure of hormonal support appears to be a major cause of habitual abortion Hence progestational therapy is indicated in all women who have a history of abortion or who are pregnant and threatening to abort The fetal salvage results obtained with Delalutin for practical purposes are the same as those obtained with free progesterone Therefore because of the ease and less frequency of administration and the freedom from reactions even at high doses the caproate ester is obviously the therapeutic agent of choice in classical habitual abortion The same conclusion applies logically to the treatment of women with less than three recurrent abortions and to patients with threatened abortion

At the present it is our impression that a total of at least 7 g of Delalutin is needed during the course of the gestation in patients with habitual abortion The best response seems to occur when treatment with the steroid ester is started as soon as possible after the onset of therapy, and when a single injection of 250 to 500 mg is administered once each week until the patient is within 14 days of the expected or the proposed date of delivery If treatment is started early and continued regularly to term a dosage of 250 mg per week may prove to be satisfactory therapy in many patients with *classical habitua abortion* and in most women who have had *less than* three *consecutive interrupted pregnancies* Since prompt and vigorous treatment is indicated for *threatened abortion* a single injection of 500 mg or more is suggested every 2 or 3 days until symptoms subside and have been absent for 10 days

SUMMARY AND IMPRESSIONS

1 *Failure of hormonal support* is a major factor responsible for classical habitual abortion in man The administration of adequate amounts of progestational therapy throughout pregnancy in patients with this condition results in a *more than three fold increase* in the number of babies that are saved

2 *17 α hydroxyprogesterone caproate Delalutin* * because of its high potency, prolonged action and freedom from reactions is *the preferred agent* for treating patients (1) with habitual abortion (2) with less than three con secutive abortions and (3) with threatened abortion

3 The current data suggest that *adequate Delalutin therapy* for habitual abortion is

(*a*) a total of 7 g *administered during the course of the gestation*
(*b*) treatment started as soon as possible *after the onset of pregnancy*
(*c*) treatment continued to within two weeks of the expected or proposed date of delivery and
(*d*) a single injection of 250 to 500 mg once a week

4 A single injection of 250 mg of Delalutin once a week may prove to be satisfactory therapy in most women who have had *less than three consecutive interrupted pregnancies*

5 For *threatened abortion* a single injection of 500 mg or more of Delalutin is suggested every 2 or 3 days until symptoms subside and have been absent for 10 days

6 *When too little Delalutin is given* in habitual abortion it appears that undesirable events may occur

(*a*) moderately suboptimal dosage started between the 6th and the 7th week and terminated before the 28th week of gestation often is fol lowed by *premature labor*
(*b*) markedly suboptimal dosage started after the 5th week of gestation often fails to prevent the death and simultaneous expulsion of the products of conception (*abortion*) and
(*c*) markedly suboptimal dosage started before the 5th week of gestation often is followed by death of the products of conception and delayed expulsion with *missed abortion*

7 *17 α hydroxyprogesterone caproate* (*Delalutin*) *is a potent long acting progestational agent* which has an important role in the therapy of human obstetrical complications particularly *recurrent abortion* It should be evaluated as a therapeutic agent in the management of repeated abortion in domestic animals

Acknowledgment—The author gratefully acknowledges the co operation of the following investigators who have made their data available for this com pilation Drs W M Allen S J Behrman M R Cohen R R Commons J T Cowan D M Fahrenbach J J Gold L F Hawkinson C P Hodgkinson G W Hunter B D Jacobson G E Seegar Jones A H Marbach M S Margolese J B McCoy Jr E W Page C J Podore

* Delalutin E R Squibb & Sons New York

B B Rubenstein W R Schuman S G Taylor III H H Thomas E T Tyler and L Wilson The author also is indebted to Audrey Randall and her staff of secretaries and to Marie Schumann Helen MacIvor Margaret Smiles and D R Zimmerman for assistance in preparing the manuscript, and particularly to Don Forer and Paul Lawler for making the charts

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ESTROGEN DETERMINATION IN BLOOD AND BODY FLUIDS OF CATTLE

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THE object of our investigations has been to develop a routine chemical procedure for the determination of estrogens in the blood and body fluids of dairy cattle The studies contained in this report may be grouped broadly into two sections

1 The determination of estrogens in blood urine colostrum and follicular fluid
2 The influence of impurities in biological extracts on the chemical determination of the estrogens
   A Alteration of partition chromatography characteristics
   B Quenching of fluorescence of the estrogens

MATERIALS AND METHODS

Partition chromatography on a Celite–NaOH column by the method previously described (Bitman and Sykes 1953) was used to separate estrone estradiol and estriol One modification in the procedure consisted of transferring the estrogen containing residue to the column in 1 0 ml of alcohol–toluene (1 19) rather than in 1 0 ml of benzene

Fluorescence was measured by the procedure of Engel *et al* (1950) in a Coleman Model 12 photofluorometer with a lamp filter transmitting at 436 mμ (Corning No 3389 and Corning No 5113 glass filters) and photocell filter transmitting at 488 mμ (Baird 488 mμ interference filter and Corning No 3387 glass filter)

The biological activity of the extracts was estimated by the 6 hour uterine weight method of Astwood (1938) Each extract was dissolved in olive oil and 0 1 ml injected subcutaneously in 20–24 day old Osborn female rats weighing between 39 and 47 g Four to ten animals were used for each determination Dose response curves were established for our colony for estradiol estrone estriol estradiol benzoate and estrone sulfate standards

The estrogen fraction was separated from the neutral fraction of the extracts by the procedures of Engel *et al* (1950) and Friedgood Garst *et al*

(1948) The residue from an extract containing the estrogens and the neutral steroids was dissolved in 95 ml CCl_4–ether (18 1) and the solution extracted 1 × 100 ml 2 × 50 ml N KOH The CCl_4–ether was washed 2 × 5 ml water and the washings added to the KOH phase The KOH was adjusted to pH 9 0 ± 0 5 with 6 N H_2SO_4 2 ml of Engel concentrated buffer (20 volumes saturated $KHCO_3$ and 1 volume saturated K_2CO_3) were added and the solution extracted 4 × 50 ml ether The ether solution was dried over Na_2SO_4 and evaporated to dryness under reduced pressure

In order to remove lipid material from the extracts, the residues were dis solved in 50 ml 70% ethanol and the solution extracted 2 × 50 ml Skelly solve B (lipid fraction discarded) Twenty milliliters of distilled water were added to the ethanol to make a phase consisting of 50% ethanol It was found advantageous to extract against 50% ethanol since only 1 volume of ether was needed to pro duce a complete phasic separation of ether–ethanol vs water whereas 4–5 volumes of ether were necessary against 70% ethanol The 50% ethanol was then extracted 2 × 100 ml ether The lower phase consisted of 35 ml water while the upper phase of ether–ethanol contained the estrogens The ether–ethanol was distilled off under reduced pressure This procedure was tested independently with estrone estradiol and estriol and they were quantitatively recovered in the ether–alcohol phase

Unless otherwise noted all extracts which have been chromatographed on the Celite–NaOH partition column have been purified via the CCl_4–ether–KOH procedure to remove neutral steroids and via the lipid removal procedure

1 *The Determination of Estrogens in Blood Urine Colostrum and Follicular Fluid*

After the development of a satisfactory partition chromatographic method which would effectively separate pure estrone estradiol and estriol (Bitman and Sykes 1953) the application of this method to suitable extracts of blood was next investigated Szego and Roberts (1946 1947 Roberts and Szego 1946) using bioassay methods reported concentrations of 3 0 to 8 0 μg of β estradiol equivalents per liter of blood Extracts were prepared according to their methods and the material so obtained was subjected to our chromato graphic and fluorimetric procedures Several of the chromatographic fractions exhibited fluorescence but were not biologically active when tested by the 3 day uterine weight method (Lauson *et al* 1939) on ovariectomized 21 day old rats

Since the Szego and Roberts procedure in our hands yielded extracts with fluorescence but with no biological activity it seemed necessary to obtain extracts which satisfied both of these criteria i e fluorescence and biological activity before we could attribute the fluorescence obtained to the presence of estrogens The non-correlation of fluorescence and biological activity demon strated the danger in reliance upon fluorescence alone We therefore attempted

to prepare extracts which would be biologically active The 6 hour Astwood uterine weight bioassay was used in this phase of the study This permitted work up of a smaller amount of blood than that needed for the 3 day uterine weight method It was found possible to prepare an extract and test it on the following day The variations in the extraction procedures which we made were based on the possible forms in which estrogen may exist in blood These possibilities are (1) free estrogen (2) estrogen-conjugate (3) estrogen protein (4) estrogen conjugate protein Approximately fifteen variations have been attempted Most of these procedures fall into four main categories

A Dialysis subsequent hydrolysis and extraction
B Direct extraction of free estrogens
C Direct hydrolysis extraction of the liberated estrogens
D Direct extraction of free and conjugated estrogens subsequent hydrolysis and extraction

(A) *Dialysis of the Estrogens from Blood*

Two dialysis procedures were used that of Szego and Roberts (1946) and the method reported by Zaffaroni (1953) In the Szego and Roberts procedure whole blood or plasma from pregnant cows was dialyzed against distilled water for 48–96 hours In the Zaffaroni method the blood or plasma was diluted with equal volumes of water and methyl alcohol and dialyzed against 40% methanol for 48–96 hours The results of dialyzing blood or plasma from pregnant cows are shown in Table I Included are data on uterine weights of control rats and rats injected with estradiol In two cases (exp 2 7) positive biological activity was obtained with the Szego and Roberts technique When four other samples of pregnancy blood (exp nos 3 4 5 6) from the same cow (N293) were tested no estrogenically active material could be obtained The other biologically active sample was obtained from cow N646 A previous sample (exp 1) of whole blood was inactive another sample of plasma treated by the Zaffaroni technique was inactive (exp 8) The proteins obtained from blood and colostrum by precipitation with MeOH were dialyzed by the Zaffaroni method (exp 15 16) No biological activity was observed

The colostrum samples exhibited estrogenic activity of 1 0 μg 1 75 μg and 1 75 μg equivalents of β estradiol per liter

Samples of pregnancy plasma from all cows except one were negative (15 samples from 13 different cows) The samples from one cow (No 298) consistently showed biological activity (exp nos 5 6 7 8 and 9) During the next pregnancy of this cow one sample of plasma was negative while another showed borderline activity (exp nos 23 and 24) Samples of blood from a cow in heat and from an abnormally cycling cow were worked up using this procedure One sample (exp no 27) from the abnormally cycling cow was positive at a low level of estrogenic activity Another sample showed no activity

Samples of follicular fluid from two nymphomaniac cows with ovarian

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TABLE II

Extraction of Estrogens from Body Fluids with MeOH

Exp No	Fluid	Cow No	Average uterine weight (mg)		Estrogen as β-estradiol
			Free	Conj	μg/l
1	Colostrum	10	21 6	30 9	1 0
2	Colostrum, sample A	11	—	31 5	2.0
	Colostrum, sample B	—	—	30 9	1 5
3	Colostrum sample A	12	—	35 2	1 5
	Colostrum, sample B	—	—	34 3	2.0
4	Plasma—pregnancy 6–8 months	294	24 3	21 9	—
5	Plasma—pregnancy 6–8 months	298	—	29 1	0 8
6	Plasma—pregnancy 6–8 months	298	—	25 7*	—
7	Plasma—pregnancy 6–8 months	298	—	28 8	0 6
8	Plasma—pregnancy 6–8 months	298	—	28 1	0 5
9	Plasma—pregnancy 6–8 months	298	—	28 7	0 8
10	Plasma—pregnancy 6–8 months	829	—	25 3*	—
11	Plasma—pregnancy 6–8 months	184	—	24 2	—
12	Plasma—pregnancy 6–8 months	184	22.6	21 8	—
13	Plasma—pregnancy 6–8 months	184	—	24 8	—
14	Plasma—pregnancy 6–8 months	829	—	23 6	—
15	Plasma—pregnancy 6–8 months	186	21 5	21 7	—
16	Plasma—pregnancy 6–8 months	2886	24 4	23 6	—
17	Plasma—pregnancy 6–8 months	832	22 0	25 9*	—
18	Plasma—pregnancy 6–8 months	830	21 5	22.9	—
19	Plasma—pregnancy 6–8 months	331	22 5	—	—
20	Plasma—pregnancy 6–8 months	331	22.6	—	—
21	Plasma—pregnancy 6–8 months	835–838	20 7	20 6	—
22	Plasma—pregnancy 6–8 months	837–1018	22 8	23 8	—
23	Plasma—pregnancy 6–8 months	298	22.4	23 7	—
24	Plasma—pregnancy 6–8 months	298	25 8*	25 9	—
25	Plasma—during estrus	298	—	21 5	—
26	Plasma—irregular estrus cycle	2414	19 9	21 5	—
27	Plasma—irregular estrus cycle	2414	—	27 4	0 3
28	Follicular fluid—nymphomaniac	803	35 3	23 0	79 0
29	Follicular fluid—nymphomaniac	466	33 0	23 2	29 5

A 25 5 mg uterus has been taken to represent the threshold value for the Astwood uterine weight assay with our colony Uteri of this weight are in the borderline range o activity and their significance is difficult to assess

estrogens showed 79 0 μg/l and 29 5 μg/l of β-estradiol equivalents Fluorescence indicated 126 μg/l and 62 μg/l respectively All other fractions of the free estrogens were negative and showed little fluorescence

The conjugated fractions both showed fluorescence in the earliest eluate the fore run fraction which calculated as 36 μg/l (803) and 32 5 μg/l (466) of estrone When tested biologically one fraction (803) was negative while the other (466) showed 8 9 μg/l of β-estradiol equivalents or 49 0 μg/l of estrone equivalents

TABLE I

Dialysis of Estrogens from Blood

Exp	Treatment	Cow No	Equivalent dose (ml blood/rat)	Dialysis temp C	Average uterine weight in mg±S E	β-Estradiol equivalents μg/1
	Szego and Roberts Method					
1	Blood vs H_2O 48–96 hours	646	20	0–5	22 2±0 89	—
2	Blood vs H_2O 48–96 hours	293	29	0–5	27 9±0 70	3 1*
3	Blood vs H_2O 48–96 hours	293	38	0–5	22 5±0 59	—
4	Blood vs H_2O 48–96 hours	293	33	0–5	20 9±0 76	—
5	Blood vs H_2O 48–96 hours	293	33	0–5	23 7±1 28	—
6	Blood vs H_2O 48 96 hours	293	25	25	21 7±0 87	—
7	Plasma vs H_2O	646	33	25	27 1±1 82	0 7
	Zaffaroni Dialysis Method					
8	Plasma H_2O MeOH (1 1 1) vs 40% MeOH	646	33	25	22 1±1 28	—
9	Blood H_2O MeOH (1 1 1) vs 40% MeOH	1003	17	25	23 3±1 40	—
10		294	33	25	19 8±1 56	—
11		87	33	25	22 3±0 24	—
12		829	100	25	24 9±1 24	—
13		829	50	25	23 4±2 30	—
14		829	85	25	24 6±0 25	—
15	Blood proteins 33% MeOH vs 40% MeOH	331	100	25	22 1±1 40	—
16	Colostrum proteins 33% MeOH vs 40% MeOH	331	75	25	24 0±1 88	—
17	Control group				21 8±0 23	
18	Estradiol standards	0 0125 μg			25 7±0 82	
		0 025 μg			27 5±0 75	
		0 050 μg			31 7±0 59	
		0 075 μg			35 5±1 17	

A straight line relationship holds between uterine weight and dosages of β-estradiol from 0 0125 μg to 0 10 μg With dosages below 0 0125 μg variations in response are great and results are meaning less A 25 5 mg uterus has therefore been taken to represent the threshold value for the Astwood assay with our rat colony Uteri of this weight are in the borderline range of activity and their significance is difficult to assess

* Significant at 1 % level (Fisher s t test)

cysts were obtained at slaughter and processed according to the Pope and Roy procedure (Table II Nos 28 and 29) The free and conjugated fractions were chromatographed on the Celite partition column and each fraction was assayed biologically and fluorometrically The results are shown in Table III Both samples showed essentially similar patterns The estradiol fraction of the free

TABLE II

Extraction of Estrogens from Body Fluids with MeOH

Exp No	Fluid	Cow No	Average uterine weight (mg)		Estrogen as β-estradiol
			Free	Conj	μg/l
1	Colostrum	10	21 6	30 9	1 0
2	Colostrum, sample A	11	—	31 5	2 0
	Colostrum sample B	—	—	30 9	1 5
3	Colostrum sample A	12	—	35 2	1 5
	Colostrum sample B	—	—	34 3	2 0
4	Plasma—pregnancy 6–8 months	294	24 3	21 9	—
5	Plasma—pregnancy 6–8 months	298	—	29 1	0 8
6	Plasma—pregnancy 6–8 months	298	—	25 7	—
7	Plasma—pregnancy 6–8 months	298	—	28 8	0 6
8	Plasma—pregnancy 6–8 months	298	—	28 1	0 5
9	Plasma—pregnancy 6–8 months	298	—	8 7	0 8
10	Plasma—pregnancy 6–8 months	829	—	25 3*	—
11	Plasma—pregnancy 6–8 months	184	—	24 2	—
12	Plasma—pregnancy 6–8 months	184	22 6	21 8	—
13	Plasma—pregnancy 6–8 months	184	—	24 8	—
14	Plasma—pregnancy 6–8 months	829	—	23 6	—
15	Plasma—pregnancy 6–8 months	186	21 5	21 7	—
16	Plasma—pregnancy 6–8 months	2886	24 4	23 6	—
17	Plasma—pregnancy 6–8 months	832	22 0	25 9	—
18	Plasma—pregnancy 6–8 months	830	21 5	22.9	—
19	Plasma—pregnancy 6–8 months	331	22 5	—	—
20	Plasma—pregnancy 6–8 months	331	22 6	—	—
21	Plasma—pregnancy 6–8 months	835–838	20 7	20 6	—
22	Plasma—pregnancy 6–8 months	837–1018	22 8	23 8	—
23	Plasma—pregnancy 6–8 months	298	22 4	23 7	—
24	Plasma—pregnancy 6–8 months	298	25 8	25 9*	—
25	Plasma—during estrus	298	—	21 5	—
26	Plasma—irregular estrus cycle	2414	19 9	21 5	—
27	Plasma—irregular estrus cycle	2414	—	27 4	0 3
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TABLE III

Fluorescence and Biological Activity of Chromatographic Fractions from Follicular Fluid

Cow No	Fraction	μg equivalents per liter in column fractions									
		Fore run		Estrone		Interzone		Estradiol		Estriol	
		Biol	Fluor	Biol	Fluor	Biol	Fluor	Biol	Fluor	Biol	Fluor
803	Free	0	0	0	0	0	0	79 0	126	0	0
	Conj	0	36	0	0	0	0	0	0	0	0
466	Free	0	0	0	0	0	0	29 5	62	0	0
	Conj	8 9*	32 5	0	0	0	0	0	0	0	0

* As β estradiol equivalents.

These results are in agreement with the recent data of Duncan *et al* (1955) who reported 130 μg/l of β estradiol for follicular fluid and 86 μg/l for an ethanol extract of the fluid No fractionation of the estrogens was reported

Since the methanol extraction procedure gave biological activity with colostrum and follicular fluid but yielded negative results in almost all samples of bovine blood assayed it seemed desirable to test the procedure further with samples of human blood known to contain estrogens Accordingly several 100 ml samples of blood were obtained from a human patient 1 or 2 hours after the intravenous administration of 100 mg doses of Premarin Four samples were worked up through the methanol procedure and the fractions chromatographed and assayed fluorometrically and biologically Almost similar qualitative patterns were obtained Estrogenic activity was generally found in the fore run and/or estrone eluates of the conjugated estrogen fractions This was the expected finding since Premarin is mainly composed of estrone sulfate Results are shown in Table IV Reasonably close correspondence between the estrogen content as calculated from fluorescence and from bioassay data was obtained particularly where larger amounts of biological activity were found The values however were considerably lower than the estrogen content obtained 6 years previously when the samples were fresh and were assayed directly by injection of the serum into ovariectomized rats the samples were frozen during the period 1949–1955

Two experiments were conducted to test the procedure on the recovery of estrogens in bovine blood In both trials 200 mg of 17β estradiol 3 monobenzoate was injected intravenously into cow N2414 Pre-injection and 1 hour post injection blood samples were taken and worked up by the Pope and Roy procedure In experiment 1 the free fraction contained 18 8 μg/l of β estradiol equivalents in experiment no 2 conducted 2 months later the free fraction contained 40 0 μg/l of β estradiol equivalents This was equivalent

TABLE IV

Estrogen Content of Human Blood from Patient after Administration of 100 mg Premarin

Sample No	Fraction	µg equivalents per ml									
		Fore-run		Estrone		Interzone		Estradiol		Estriol	
		Biol	Fluor	Biol	Fluor	Biol	Fluor	Biol	Fluor	Biol	Fluor
1	Free	0	0 7	0	0	0	0	0	0	0	1 4
	Conj	0	0	5 25	5 5	0	0	0 75	0	0	0
2	Free	0	0 8	1 9	0	1 9	0	0	0	0	1 7
	Conj	0	0 7	3 8	0 7	0	0	0 2	0	0 2	0 8
3	Free	0	0 6	0	0 5	0	0	0	0	0	1 3
	Conj	4 5	5 0	9 0	6 0	0	0	0	0	0	0
4	Free	0	0 8	0	0 8	0	0	0	0	0	1 5
	Conj	2 5	3 3	0	2 5	0	0	0	2 1	0 4	2 9

to 1 3 mg and 2 8 mg of β estradiol in the total calculated blood volume of the cow 1 hour after the injection of 200 mg of estradiol benzoate (equivalent to 145 mg of 17β estradiol) and represented recoveries of 0 90% and 1 93% respectively Duncan *et al* (1955) found 145 µg of estrogen as β estradiol equivalents in the total calculated blood volume of a cow 30 minutes after the intravenous injection of 10 mg of estradiol This was a recovery of 1 45% of the administered dose The conjugated fractions in both of our experiments and the pre injection blood samples showed no biological activity

2 *The Influence of Impurities in Biological Extracts on the Chemical Determination of the Estrogens*

(a) *Alteration of Partition Chromatography Characteristics*

A factor influencing the interpretation of the chromatographic results is the possibility that a particular chromatographic system which effectively separates pure compounds may be altered in the presence of other substances from a biological extract Such alterations in distribution have been noted in chromatography of steroids (Dobriner Lieberman and Rhoads 1948) and in partition chromatography of steroids (Savard 1954) The usual effect is an elution of the compound in the extract by a less polar solvent than is used with the pure compound in the case of chromatography or of an earlier elution in partition chromatography In several instances the fore run and inter zone fractions exhibited fluorescence and biological activity (Tables III and IV) indicating an earlier elution of estrone and estradiol This effect was studied by observing the displacement in elution behavior of the pure steroids added to plasma

Quantities of 5 0 and 10 0 μg of β estradiol were added to 200 ml and 500 ml samples of pregnancy plasma respectively and treated according to the methanol extraction procedure described above Recoveries of 72% and 94% were obtained biologically in the free fractions The sample which had a recovery of 94% by bioassay showed a recovery of only 14 8% fluorometrically Quenching and/or self absorption in the impure brownish colored extracts indicated the need for further purification

The free fraction from this sample was therefore chromatographed on the Celite–NaOH column to purify the extract and separate the added estradiol The purification effected on the column was sufficient to enable estradiol to be determined fluorometrically Complete fluorometric recovery was obtained The fore run fractions of the control chromatogram and the estradiol added sample were assayed biologically since they exhibited fluorescence No activity was observed With extracts from biological sources i e blood or urine a yellowish zone which exhibits fluorescence has usually appeared in the fore run fraction of our chromatograms The concomitant use of bioassay and fluorometry has enabled us to attribute this fluorescence to non estrogenic materials

The fact that the estradiol which had been added to the plasma appeared in the estrone fraction and in the inter zone eluate of the chromatogram indicated that a disturbance in the partition characteristics had taken place with these extracts The estradiol fraction where the added material was expected to appear exhibited no biological activity and little fluorescence This alteration was further established by adding 5 0 μg of β estradiol to a control extract of this plasma just prior to its chromatography on the Celite–NaOH column (Table V no 3) The added estradiol eluted earlier and ap

TABLE V

Chromatography of Estradiol added to Plasma Fractions

Fraction	% recovery in column fractions									
	Fore run		Estrone		Interzone		Estradiol		Estriol	
	Biol	Fluor	Biol	Fluor	Biol	Fluor	Biol	Fluor	Biol	Fluor
1 Plasma	0	20	0	0	0	0	0	0	0	0
2 Plasma + 10 μg estradiol	0	20	60	62 5	40	47 5	0	0	0	0
3 Plasma extract + 5 μg estradiol added just prior to chromato-	0	20	106	109	10	0	0	0	0	0

peared quantitatively in the estrone fraction both fluorometrically and biologically A small amount (0 5 μg) was determined biologically in the inter zone eluate Bauld (1955) using a Celite–NaOH partition column also found a decrease in the retention volumes of estrone and estradiol in the presence of urinary chromogens

It seemed worthwhile to investigate further the column behavior in the presence of biological impurities Free and conjugated fractions were pre pared from a 3 0 l sample of pregnancy plasma Quantities of 10 μg of estrone and estradiol were added to aliquots of the free and conjugated frac tions equivalent to 500 ml of plasma and chromatographed on the Celite–NaOH column The fluorescence and biological activity of the eluted fractions are shown in Table VI

TABLE VI

Changes in Partition Chromatography of Estrogens in the Presence of Biological Extracts

N B Percentage Recovery in Column Fractions

Fraction	Fore run		Estrone		Interzone		Estradiol		Estriol	
	Biol	Fluor	Biol	Fluor	Biol	Fluor	Biol	Fluor	Biol	Fluor
1 Free	0	0	0	0	0	0	0	0	0	0
2 Free + 10 μg Estrone	0	0	100	107	0	0	0	0	0	0
3 Free + 10 μg Estradiol	0	0	0	0	30	28	70	73	0	0
4 Conjugated	0	0	0	0	0	0	0	0	0	0
5 Conj + 10 μg Estrone	100	24	0	0	0	0	0	0	0	0
6 Conj + 10 μg Estradiol	0	0	100	100	0	0	0	0	0	0

One sample of plasma (198) and two samples of whole blood (840A and 840B) were dialyzed according to the Szego and Roberts procedure in the cold room for 3–6 days The dialyzate was hydrolyzed and extracted according to their procedure and the resulting extract chromatographed on the Celite partition column No biological activity was obtained in the chromatographic fractions although fluorescence equivalent to 6 4 μg/l and 3 0 μg/l was obtained in the estriol fractions in 2 of the 3 samples

(B) *Direct Extraction of Free Estrogen*

Veldhuis (1953) recently described a method for the chemical determination of estrogens in plasma based on fluorometry of ether extracts of unhydro lyzed plasma Fluorescence corresponding to 31 to 49 μg/l of unconjugated

estrogens was obtained in the three samples of human pregnancy plasma he studied The Veldhuis procedure was followed exactly with plasma from preg nant cows up to the point of the separation of the estrone–estradiol and estriol fractions In one case borderline activity (calculated to be 0 2 μg/l o β estradiol equivalents an average uterine weight of 25 6 mg for the test group) was observed This same sample gave a fluorescence value of 25 μg/l Two other fluorescent samples gave no indication of estrogenic activity on bioassay

Two 600 ml samples of pregnant cows plasma were processed in the Veldhuis procedure as outlined above and chromatographed Sample 198 prior to chromatography gave a fluorescence value of 7 8 μg/l calculated as estrone the fluorescence of sample 840 calculated as 14 1 μg/l of estrone Biological activity was found only in the estrone fractions of both plasma samples corresponding to 0 5 μg and 0 4 μg β estradiol equivalents/l The fore run and estriol fractions both showed fluorescence but no biological activity thereby serving as a reminder of the non specificity of the fluoro metric method Veldhuis has estimated the non specific fluorescence by de stroying the estrogen fluorescent compound with hydrogen peroxide and measuring the residual non specific fluorescence He found that non specific fluorescence was 24% 71% and 82% of the total fluorescence in the estriol fractions and 18% 21% and 25% of the total in the estrone–estradiol fractions

In a study of urinary estrogens using countercurrent fractionation, Migeon (1953) found that the non specific fluorescence was 70% of the total for urinary extracts from normal adults and 25–30% of the total for pregnancy urine extracts

The data presented here also indicate a large amount of non specific fluorescence since the fluorescent fractions show little if any biological activity Physiological specificity of an organ or tissue is still in most instances much greater than that which can be achieved with physical or physico chemical procedures notwithstanding a variety of solubilities reactions or optical systems

(C) *Direct Hydrolysis and Extraction of the Liberated Estrogens*

Preliminary attempts to hydrolyze the conjugated estrogens in blood and at the same time release them from protein conjugates by heating with HCl or H_2SO_4 in the concentrations commonly employed for urinary hydrolyses (15 vol % and 5 vol % respectively) produced troublesome foaming precipitates, suspensions and gels The emulsions that form when organic solvents are added also offer considerable difficulties which must be overcome in preparing extracts

A concentration of 35% H_2SO_4 (54 vol %) has been used to completely hydrolyze proteins (Schmidt 1938) and when this concentration is effected

with plasma or blood the physical troubles described above can be avoided During the 10 minute heating period the foaming can be controlled by the addition of a small amount of Dow Corning Antifoam AF Emulsion This silicone compound exhibits no fluorescence or estrogenic activity It does not quench the fluorescence or inhibit the biological activity of the pure estrogens Ether extraction can be carried out with little difficulty since the emulsions which form are not stable Since this is a much higher concentration of acid than is usually used the effect of this treatment on small amounts of pure estradiol was determined

After heating 10 μg of estradiol in 54% (vol %) H_2SO_4 for 10 minutes a 40% loss in biological activity was observed No loss in fluorescent intensity occurred however This difference between the partial recovery of biological activity as compared with the complete recovery of fluorescence may be explained by an alteration of 40% of the estradiol in such a manner that the structures necessary for biological activity were not present while those portions of the estrogen molecule necessary for fluorescence remained intact

Katzman *et al* (1954) have recently studied the effect of refluxing pure estrogens in aqueous solutions with 15 vol % HCl for periods ranging from 10 to 60 minutes No losses were observed when determined by the Kober reaction Similar experiments carried out by workers using bioassay resulted in marked losses in activity (Smith and Smith 1941 Van Bruggen 1948) Katzman emphasizes the possibility that alterations might have occurred even though those structures necessary for color development with the Kober reagent were maintained Boscott (1949) found that changes in the absorption characteristics of estradiol occurred after refluxing in aqueous solution with 15 vol % HCl

When 10 μg of estradiol were added to plasma and heated with 54% H_2SO_4 only 40% of the activity could be recovered biologically In spite of and considering these losses this procedure was applied to the plasma of pregnant cows All samples tested were negative

Since the plasma proteins were responsible for many of the practical difficulties of extraction attempts were made to hydrolyze them prior to the acid hydrolysis of the conjugated estrogens Strong alkaline hydrolysis (heating plasma with 16 5 N KOH followed by hydrolysis with 15 vol % HCl) trypsin hydrolysis followed by glucuronidase treatment or glucuronidase treatment alone all failed to yield estrogenically active extracts Treatment of precipitated plasma proteins with 54% H_2SO_4 or 15 vol % HCl also gave negative biological results Dilution of the plasma with 1 volume of ethanol followed by hydrolysis with HCl was ineffective as was HCl hydrolysis alone

Several urine samples from pregnant cows were hydrolyzed with 15 vol % HCl extracted with ether and the phenolic extract chromatographed

Results are shown in Table VII Some degree of correlation was found between biological activity and fluorescence Thus, the estrone fraction of cow 331 showed 51 3 µg biologically and 35 9 µg fluorometrically and the estrone fraction of cow 828 contained 227 µg biologically and 198 µg fluorometrically

TABLE VII

Fluorescence and Estrogenic Activity of Chromatographic Fractions of Urinary Extracts

Cow No	µg equivalents/l in column fractions									
	Fore run		Estrone		Interzone		Estradiol		Estriol	
	Biol	Fluor	Biol	Fluor	Biol	Fluor	Biol	Fluor	Biol	Fluor
331	39 0	0	51 3	35 9	0	23 0	12 1	42 6	0	0
828	140	113	227	198	0	0	0	17	0	0
298	64 8	—	68 4	—	0	—	0	—	0	—

Some correspondence was found in other fractions In certain fractions, however biological activity was obtained with no fluorescence and conversely fluorescence was exhibited although estrogenic activity was non existent A similar lack of specificity in the fluorometric estimation of estrogens in cow urine has been recognized by Smith Dickson and Erb (1956) These trials also suggested that impurities in the urinary extracts had altered the distribution of estrogens on the chromatogram since estrogenic activity was found in the fore run fractions in all three samples

(D) *Direct Extraction of Free and Conjugated Estrogens followed by Hydrolysis and Extraction*

Direct extraction of the blood or plasma with methyl alcohol was next attempted Pope and Roy (1953) in a recent investigation of the estrogenic activity of bovine colostrum extracted colostrum with boiling methanol Since colostrum at least with regard to its protein content is much like plasma it was decided to apply their method to plasma As a preliminary three samples of bovine colostrum were assayed for their estrogenic activity These results are shown in Table II along with the application of this procedure to blood and follicular fluid

Contrary to the results of the previous experiment there was little disturbance in the distribution of the estrone and estradiol which had been added to the free fraction of the plasma Estrone was eluted in the expected fraction but estradiol was eluted somewhat earlier 28% appearing in the inter zone fraction When added to the conjugated fraction of the plasma and chromatographed both estrone and estradiol were eluted much earlier Estrone was

found in the fore run and estradiol in the estrone eluate The control conjugated fraction showed the fluorescence usually seen in the fore run fractions The estriol fraction also exhibited fluorescence Neither of these fractions exhibited biological activity The sample to which estrone was added yielded an amount of fluorescence equivalent to a recovery of only 24% Bioassay showed that 10 μg of estrone was present in this fraction thereby indicating that the remaining three quarters of the fluorescence due to the estrone had been quenched No quenching of the estradiol was observed and complete recoveries were obtained biologically and fluorometrically from the estrone fraction in which it was eluted It thus appears that the yellowish zone which usually is eluted in the fore run fraction is capable of quenching the fluorescence of estrone to a marked degree The elution of estriol has not been affected by the impurities present in the extracts Bauld (1955) has reported a similar finding

The alteration of the partition characteristics of the column was variable in the presence of the impurities in the extracts It seems necessary therefore to effect further purification of the extracts prior to chromatography to remove these substances which influence the elution pattern in studies of this kind In lieu of this it would appear that standard columns on which pure estrogens are chromatographed in the presence of biological extracts must be run if reliable interpretations of the chromatographic patterns are to be made

(B) *Quenching of Fluorescence of the Estrogens*

The finding of high biological activity but little fluorescence in many of the chromatographic fractions could be due to quenching of the fluorescence of the estrogens by other components of the fraction An attempt was therefore made to dilute the quenching agent sufficiently to enable the fluorescence of the sample to be measured Accordingly several aliquots of chromatographic fractions which showed this behavior were treated in the normal fluorometric procedure and the fluorescence reading recorded They were then diluted with an amount of 65% H_2SO_4 equivalent to the total volume (8 2 ml) in the fluorometer tube mixed thoroughly and the fluorescence reading again determined A third dilution was made in one instance by adding an additional 8 2 ml of the diluent acid When standards are diluted the fluorescence decreases as expected in almost direct relation to the quantity of diluent added Table VIII shows results of dilution of the quenching substances in chromatographic fractions In several instances (1 2 4) a 2 × dilution produced increases in the fluorescence of the sample In several other cases (3 5 6 7 8) the fluorescence decreased but did not do so proportionally as the standards did Considering the volume changes these increases range from 1 5 to 11 times the original fluorescence

Quenching is a factor which must be taken into consideration when using a fluorescent method on biological extracts The validity of this method of elimi

TABLE VIII

Dilution of the Quenching Agent in Fluorescent Solutions

Fraction	Fluorometer reading		
	Original (8 2 ml)	2 × Dilution (16 4 ml)	3 × Dilution (24 6 ml)
1 1B1	11	29	37
2 RE1B1	70	100	—
3 1B4	36	26	—
4 1B1C1	88	94	—
5 1B2C	100	66	—
6 198	22	17	—
7 840	42	33	—
8 840	36	28	—
Estrone 2 0 μg	96	54	—
1 0 μg	52	26	—
Estradiol 2 0 μg	62	34	—
1 0 μg	32	18	—
Estriol 2 0 μg	62	32	—
1 0 μg	30	16	—
Blank	6	6	4

nation of the quenching action has not been established Other correction procedures are (1) the determination of fluorescence of aliquots of extracts with and without the addition of estrogen standards (Finkelstein, 1952) and (2) the application of correction equations (Braunsberg Osborn and Stern 1954)

SUMMARY

Several procedures based on the possible forms in which the estrogens might exist in blood have been applied to the determination of the estrogens in bovine pregnancy blood Only in a few instances did the resultant extracts exhibit biological activity usually at a low level (0 4 to 0 7 μg β estradiol equivalents per liter) Szego and Roberts (1946) have reported values as β estradiol approximately 5–10 times higher for normal and pregnant cows (3 0–8 0 μg β estradiol equivalents per liter) Duncan *et al* (1955) were not able to detect estrogens in the blood of estrus and non estrus cows using either the dialysis or the acetone precipitation methods of Szego and Roberts

We have consistently been successful in detecting biological activity in colostrum urine and follicular fluid of the cow and in bovine and human blood after the administration of estrogens Our methods of extraction separation and estimation are able to detect 0 2–0 3 μg of β estradiol l of blood or fluid

The conjugated estrogen fraction of three colostrum samples contained 1 0 1 75 and 1 75 μg β-estradiol equivalents/l Pope and Roy (1953) found 5 2 μg estradiol/l in the colostrum from one cow

Two samples of follicular fluid from nymphomaniac cows (polycystic ovaries) showed biological activity and fluorescence in the estradiol fraction equivalent to 79 0 μg and 29 5 μg β estradiol per liter of fluid This value agrees closely with that reported by others (Duncan *et al* 1955)

Urine samples from pregnant cows exhibited much higher amounts of fluorescence and biological activity equivalent to 100–400 μg estrogens/l

In the presence of biological impurities variations in chromatography may be encountered This alteration of the partition characteristics was variable but may be studied by running standard columns on which pure estrogens are chromatographed in the presence of the impurities Further purification of the extracts to obviate this difficulty would be desirable

Quenching of the fluorescence of the estrogens has been noted in the presence of certain biological impurities A valid quantitative method for elimination of this quenching has not been found

Unequivocal identity of the estrogens in biological materials by chemical means is difficult due to the low concentration of the steroid in the presence of high concentrations of extraneous impurities The concomitant use of bioassay and fluorescence has proved invaluable in the preceding studies In view of the many possible pitfalls such as quenching changes in partition chromatography the exhibition of fluorescence but no biological activity etc a biological material should satisfy both of these criteria before it can be termed an estrogen

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ESTROGENIC STEROIDS IN SWINE PREGNANCY URINE*

H E Bredeck† and D T Mayer
University of Missouri
(Department of Agricultural Chemistry in co-operation with Animal Husbandry Department)

INTRODUCTION

The importance of a comprehensive understanding of the physiological process of reproduction both from the academic and commercial aspects needs no reiteration As a possible means of gaining insight into this extremely complex process the pattern of estrogen excretion via the urine has been extensively studied in a variety of animals Man most domestic farm animals and several laboratory animals have been used One domestic farm animal which seems to have been almost completely neglected in this type of study is the pig The fact that Squiers *et al* (1952) reported a 35% embryonic death loss in swine emphasizes not only the financial loss to swine breeders but also the need for further research in this species The following work was concerned with the determination of estrone estradiol and estriol and was done with the hope that some particularly critical phase of the gestation period might mirror itself in the pattern of estrogen excretion in the urine and thereby indicate an area for further concentrated research

MATERIAL AND METHODS

The urine used in the following experiments was obtained at early morning collections from 4 purebred Hampshire sows This type of collection was chosen because it had been previously reported by Glasgow (1953) working on progesterone metabolites in sow urine and suggested by Holm and Firch (1955) who used bovine urine that early morning urine contained the greatest concentration of steroids All sows were bred within three days of each other Analyses were made on pooled urine samples which were obtained by mixing together 100 ml of urine from each sow From this composite sample a 100 ml aliquot was withdrawn for analysis

The methods of acid hydrolysis and extraction used were essentially those of Finkelstein (1952) with the exception that a larger sample 100 ml urine was

Approved for publication by the Director of the Missouri Agricultural Experiment Station as journal series no 1766

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employed Following hydrolysis the sample was divided into two equal portions and each part analyzed separately for estrogens

The hydrolyzed sample was diluted to 100 ml, saturated with sodium chloride (27 g) and extracted three times with 100 ml of anhydrous ether per extraction The ether extracts were pooled, washed once with 20 ml of saturated sodium bicarbonate solution and once with 20 ml of distilled water These washings were discarded and the ether phase extracted three times with 150 ml of 1 N sodium hydroxide solution The alkaline extracts were combined chilled in an ice bath brought to pH 8 5 with hydrochloric acid and extracted three times with 250 ml of ether These ether extracts were pooled washed once with 20 ml of distilled water and distilled to dryness The dry residue was taken up in 1 ml of 95% ethyl alcohol 20 ml of benzene added and the solution transferred to a separatory funnel Next 20 ml of petroleum ether (b p 34 5–55 0 C) was added and the resulting phase extracted twice with water once with an equal volume of water and once with half its volume

At this point estriol would be removed into the water phase leaving estrone and estradiol in the organic solvent fraction The fraction containing estrone and estradiol was evaporated to dryness on a boiling water bath and set aside pending further isolation

The two water extracts which would contain any estriol were combined the pH adjusted to around 8 5 by the addition of 5 ml of 1/15 M disodium phosphate solution (pH 9–9 3) and extracted three times with 33 ml of anhydrous ether The ether extracts were combined washed once with 2 ml of water and evaporated to dryness The residue was taken up in 10 ml of 50% methyl alcohol and washed once with 2 ml of carbon tetrachloride Centrifugation was required to obtain a clear methanol layer after this washing with carbon tetrachloride An aliquot of 5 ml of the clear methanol layer was withdrawn evaporated to dryness and the amount of estriol present determined colorimetrically by treating the residue with Kober reagent

CHROMATOGRAPHY

Since this extraction procedure would not have separated estrone from estradiol this separation was accomplished by a partition chromatography method similar to that reported by Braunsberg *et al* (1954)

The chromatography column was prepared by thoroughly mixing 1 25 g of Celite 535 and 1 ml of 3 1 N sodium hydroxide solution with a glass rod until a damp coarse powder resulted This damp powder was packed as evenly as possible into the chromatography tube by adding small amounts and tamping down with a glass rod after each addition The length of the column was usually 45 mm ±6 mm and the chromatography tube had an internal diameter of 9 mm The packed tube was jacketed in a water condenser which maintained a temperature of 21 ±1 C throughout the separation A 5 ml portion of a mixture of 2 parts benzene and 1 part petroleum ether (b p

60 0–90 0 C) was then transferred to the column and forced through under pressure by means of a bulb and air reservoir until the meniscus almost reached the top of the Celite Care was taken to deliver the liquid just above the Celite column This fills the column with mobile phase and removes any air from the interstices of the supporting phase It was found by measuring the volume of the eluate that approximately 2 ml of the solvent was retained by the column Equilibration between the 1 ml of sodium hydroxide solution used in packing the column and the mobile phase is unnecessary due to the relative insolubility of the two phases

The estrone–estradiol residue was taken up in 0 5 ml of the benzene–petroleum ether mixture and transferred to the column Once again the liquid was carefully delivered just above the Celite Four further transfers were made from the vessel containing the residue which removed all of the estrone estradiol present The collection of 5 ml fractions was then begun using an automatic fraction collector Pressure was applied to the top of the liquid to force it through until the meniscus reached the top of the Celite The pressure was removed 2 ml of eluting solvent added to wash down the sides of the tube and pressure reapplied Two further rinsings of the tube were carried out be fore the remainder of the solvent was added to the reservoir and forced through the column at a rate of 0 5 ml/minute A small amount of chromogenic material was eluted in the first 5 ml fraction but fortunately this fraction con tained no estrone or estradiol

After discarding the first fraction 30 ml were collected which should con tain any estrone present The receiving vessel was then changed and additional 50 ml collected which would have eluted any estradiol

The first 30 ml of eluate which contained estrone if present and the next 50 ml which contained any estradiol were reduced in volume to approxi mately 5 ml on a steam bath transferred to separate test tubes and the drying completed under reduced pressure The resulting residues were subjected to colorimetric analysis to ascertain the amount of estrone and estradiol present

QUANTITATIVE ESTIMATION

The quantitative estimation of the hormones under investigation was done colorimetrically using the Kober reagent (1931) by the modified method of Venning *et al* (1937)

Although this method works admirably with pure estrogens a major dif ficulty is encountered when it is applied to urinary extracts due to the presence of chromogens which result in a brown color Even though the interference is extremely slight in the case of estrone and estradiol due to its removal by means of chromatography it must be reckoned with particularly in the estriol fraction To do this a correction formula must be employed

The equation used in this work to compensate for the contaminating color was proposed by Allen (1950)

In order for this correction formula to have validity however the absorption spectrum of the interfering color must be for all practical purposes linear within the range under consideration

It was found using pure crystalline estrogens that the color produced with Kober reagent had an absorption maximum at 520 mμ This wave length was therefore chosen to measure the absorption and thereby estimate the estrogens quantitatively It was also necessary however to measure the absorption at two other wave lengths equidistant from the one selected in this case 520 mμ Consequently 420 mμ and 620 mμ were chosen The aforementioned requirement must now be considered In order to employ the following correction equation the absorption curve of the contaminating color must be linear between 420 and 620 mμ The curve was determined by treating the residue of an estrogen free urinary extract with Kober reagent This absorption curve as well as that of pure estrogen* treated with Kober reagent are illustrated in FIG 1 These data illustrate that the stipulation regarding the use of Allen s formula has been met and its use in this instance justified

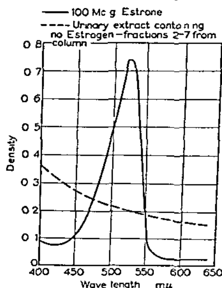


FIG 1 Absorption curves of estrone and urinary extract treated with Kober reagent.

The correction formula used was

$$CDX_x = OD_x \frac{-OD_a + OD_b}{2}$$

CDX_x = calculated density due to substance X having absorption maximum at wave length x

OD_x = observed density at 520 mμ at the absorption maximum

* The author is indebted to Parke Davis and Co and Chas Pfizer and Co Inc. for their donation of crystalline estrogens used in this investigation.

OD_a and OD_b = observed densities at 420 mμ and 620 mμ *a* and *b* must be equidistant from *x*

Thus the appropriate residues were treated with Kober reagent the color developed and the absorption measured at the three different wave lengths The observed densities were inserted into the above equation and the calculated density due to the estrogen found This calculated density was then applied to a standard curve for that estrogen and the amount of hormone read directly

RESULTS AND DISCUSSION

Analysis of the urine throughout pregnancy from four sows showed the presence of two ovarian hormones estrone and estriol This is the first time estriol has been reported in the urine of swine It has been stated by MarKee (1954) that estriol has been obtained only from human material (pregnancy urine and placenta)

The quantities found throughout gestation in the sow are summarized in Table I

TABLE I

Estrogen Excretion in Four Hampshire Sows During Pregnancy

Week of gestation	μg estrone per liter of urine	μg estriol per liter of urine
1	0	0
2	0	0
3	0	740
4	0	200
5	0	80
6	0	0
7	0	0
8	0	0
9	0	0
10	0	600
11	20	1540
12	140	1000
13	260	520
14	60	40
15	440	740

From these data it can be seen that there are two definite and distinct peaks in estriol excretion occurring around the third and eleventh week of pregnancy The estrone begins to rise around the twelfth week and after a drop reaches its maximum concentration around the fifteenth week

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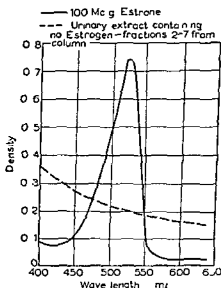


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The data presented in Table I are graphically illustrated in FIG 2 where the relationships may be more readily seen

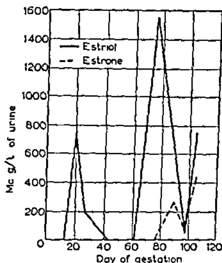


FIG 2 Estrogen excretion in four Hampshire sows during pregnancy

The presence of estrone was indicated by a positive Zimmermann (1935) reaction To verify the unexpected appearance of estriol the following experiment was carried out 100 μg of estrone and an equal amount of estradiol were taken up in the benzene–petroleum ether phase previously mentioned and partitioned twice against water This should quantitatively remove any estriol into the water phase leaving estrone and estradiol behind in the organic solvent phase When the water phase was analyzed for the presence of any hormone which in this case would represent contamination only 2 μg could be found This represents a carry over of 1% of the estrone–estradiol into the estriol fraction Therefore the color produced by the estriol fraction with Kober reagent could not be explained by estrone–estradiol contamination

In order to obtain more information about the substance giving a positive Kober reaction in the estriol fraction a number of tests were made

To obtain suitable urine the same sows were rebred after farrowing and the urine analyzed during the early part of their second gestation period Estriol however could not be found during the first four weeks of pregnancy but estrone excretion occurred during this time Collections were therefore continued until estriol was again indicated to be present during the tenth to the thirteenth week

An unknown urine extract from the eleventh week of pregnancy which previously indicated estriol to be present by a positive Kober test was used in these trials After drying the residue was spotted on paper and developed for 24 hours in an *o* dichlorobenzene–formamide system as described by Axelrod

(1953) Simultaneously five other strips of paper were developed in the same chamber They were spotted with estrone estradiol estriol a mixture of these three hormones and a blank respectively After the 24 hour period of development narrow strips of approximately 2 mm width were cut from each strip including the unknown and dipped in concentrated sulfuric acid After approximately 1 minute immersion spots became visible and when viewed under ultraviolet light they were seen to fluoresce It was observed that estriol moved 1 8–2 cm estradiol 23 5–24 5 cm and estrone had moved off of the paper The unknown extract exhibited a fluorescent spot 1 0–2 0 cm from the starting line corresponding in position to known estriol

This spot was eluted with methanol from the remainder of the strip the eluate dried and the residue taken up in a few drops of methanol and respotted on another chromatogram The unknown and two other strips containing estriol were developed for another 24 hours in a methyl*cyclo*hexane–formamide system (Axelrod 1953) After the development period narrow strips were again cut from the chromatograms and treated with sulfuric acid as before It was found that the estriol moved about 2 cm in this system and a corresponding fluorescent spot was again observed on the strip containing the urinary extract about 2 cm from the starting line

The unknown spot was eluted for a second time respotted on a third chromatogram and developed for 27 hours in an ammonium hydroxide–chloroform–benzene system according to Heusghem (1953) along with known estriol When the known estriol containing strip was immersed in sulfuric acid and viewed under ultraviolet light it was seen that the estriol had moved only 1 cm from the starting line A similarly located spot was also found on the strip spotted with the urinary extract Thus paper chromatographic evidence indicated that the substance in the estriol fraction was indeed estriol

Further evidence was obtained when this fraction after treatment with Bachman s reagent (1939) produced the typical pink color characteristic for estriol alone

It is believed that these results while not conclusively proving the presence of estriol strongly indicate its existence in the urine of swine

Thus if we consider the two hormones found in swine urine estrone and estriol their bio potency can be calculated in mouse units This has been done by considering estrone to be 16 times as active as estriol and that 1 μg of estrone is equal to 10 mouse units (Fieser and Fieser 1949)

In Table II these values were calculated and compared with figures obtained by Kust and Struck (1935) and Faiermark (1936) who used bioassay techniques

As can be readily seen there is a striking similarity between all three groups of data The difference in values occurring in the third and fourth weeks might suggest the existence of a pro estrogen or substance having biological activity not detectable chemically

TABLE II

Comparison of Estrogens found in Swine Urine during Pregnancy by Bioassay and Chemical Methods (expressed in mouse units per liter of urine)

Week of gestation	Kust and Struck (bioassay)	Faiermark (bioassay)	Chemical assay (investigation)
1	0	0	0
2	0	300	0
3	1000–2000	300	460
4	1000–2000	1000–3000	125
5	0	Drops to 1000	0
6	0	but more	0
7	0	often 300	0
8	0	↓	0
9	0		0
10	0	Rises	370
11	1000	to term	960
12	1000		2026
13	Rises		2925
14	to term	↓	600
15	5000	5–10 000	4860

The pattern of urinary estrogen excretion in humans is somewhat different from that of the sow but certain aspects are similar In humans estradiol is excreted at a fairly uniform rate throughout pregnancy with a sudden rise occurring at term The amounts of estrone and estriol constantly increase during gestation until parturition at which time estrone disappears and estriol decreases Biologic and gravimetric determinations on the excretion of these three hormones show that about 90% of the estrogenic activity in the late months of pregnancy is caused by estriol (Markee 1954)

In the case of swine no estradiol was found throughout pregnancy The total estriol was seen to rise early in gestation disappear and reappear later in pregnancy whereas estrone made its first appearance late in gestation Of course free estrone would be approximately 16 times more active than free estriol but no conclusions as to the actual proportion of the activity contributed by either one can be reached without knowing what portion of each hormone was free and what portion was conjugated

SUMMARY AND CONCLUSIONS

1 Estrogen excretion does occur through the urine in swine during pregnancy

2 The estrogenic steroids found in the urine at certain periods during pregnancy were estrone and estriol

3 There is some evidence that estrone may or may not be excreted early in gestation (during the third to the fifth week) Further work is required to

clarify this Estrone is excreted during the last fourth of the gestation period and reaches a maximum around the fifteenth week

4 Estriol excretion though possibly reaching a minor peak around the third week of pregnancy reaches its maximum concentration in the urine around the eleventh week

5 Estrogen excretion occurs at the time of placentation (during the second to the fifth week) but there is some evidence indicating that it may be estrone or estriol

6 One of the urinary estrogens was identified as estrone and its presence confirmed by a positive Zimmermann test

7 The second estrogen which possessed characteristics of estriol was established as this steroid by means of paper chromatography and the use of Bachman s reagent

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DISCUSSION

Tuesday July 2 1957

Afternoon Session

M L HOPWOOD Presiding

STEROID PHYSIOLOGY AND THERAPY

Biosynthesis of Steroid Hormones by L. T SAMUELS, Department of Biological Chemistry University of Utah Salt Lake City

The Maintenance of Human Pregnancy with Progestational Compounds by E. C. REIFENSTEIN Jr M D Associate Medical Director E. R. Squibb & Sons New York

Estrogen Determination in Blood and Body Fluids of Cattle by JOEL BITMAN T R WRENN and J F SYKES Dairy Husbandry Research Branch U S D.A. Beltsville

Estrogenic Steroids in Swine Pregnancy Urine by H E BREDECK and D T MAYER University of Missouri Department of Agricultural Chemistry

DISCUSSION

M L HOPWOOD Dr Samuel s paper is now open for discussion I have one question I would like to ask Dr Samuels Following the use of progesterone labeled C^{14} in what form is the carbon found in the aqueous fraction?

L T SAMUELS Apparently acetic acid

A V NALBANDOV Dr Samuels would you care to speculate on the possibility that the progestational activity is not due to progesterone but to the progesterone metabolites?

L T SAMUELS I think this is a definite possibility In the uterine tissue we do find definite conversion of progesterone to certain other compounds which have not been identified and it may be that this is associated with the action in those tissues

W E PETERSEN Have you any idea whether the triphosphopyridine nucleotide is synthesized locally as the result of the trophic hormone action or whether it is mobilized from other parts of the body?

L T SAMUELS Most of these tissues can synthesize the triphosphopyridine nucleotide as demonstrated by the presence of transphosphorylases

R P MARTIN Assuming this role of a trophic hormone in relation to the phos phorylation and production of the TPNH and also assuming the biosynthetic path way of androgen to estrogen would you comment on the clinical observations in relation to injections of chorionic gonadotrophin in men whereby the output of estrogen is increased considerably over that of androgen metabolites?

L T SAMUELS At the moment my only thought would be that perhaps the in creased production of TPNH enables the metabolism of the androgens to be carried more completely to the aromatic stage

I BUNDING I would like to have Dr Samuels speculate more about the nature of the action of the hormone on the intact cell as he described earlier, as opposed to the lack of action in the tissue homogenate We seem to be working ourselves down to very small molecules such as ACTH and perhaps some of the other trophic hor mones Do you feel that the action of these small molecules is localized at the cell membrane or do the molecules actually penetrate into the cells?

L T SAMUELS Whether the difference between the two is a question of the in complete system which results from destruction of some of the enzymes in the homogenates or whether the tropic hormone attacks the cell surface I would not want to say However this is an important problem to study We have of course the observations on phosphorylase A and B in regard to epinephrine action That, of course can be demonstrated in systems that have no cellular structure

M L HOPWOOD Dr Reifenstein s paper entitled The Maintenance of Human Pregnancy with Progestational Compounds is now open for discussion

F HAAG I would like to know just how soon treatment with progestational com pounds would help in preventing abortions and more particularly in preventing the loss of very early pregnancies Could you for instance start treatment immediately after estrus?

E C REIFENSTEIN Jr A number of investigators have been studying patients who ovulate but who have what is called an inadequate luteal phase These workers have started such patients on treatment with Delalutin within one week of the rise in basal body temperature which suggested that they had ovulated Some of these patients now are going through pregnancies Whether the hormone preparation had any effect, I cannot say without a much larger experience The patients referred to in my paper were individuals who had a past history of repeated abortions They were started on Delalutin treatment at the earliest on the 14th day and most of them were started at the 2nd 3rd, 4th 5th or 6th week of gestation It is my impression that the earlier the Delalutin treatment is initiated and the larger the dose the better the chances of a favorable response As I indicated we determine what is going to hap- pen toward the end of pregnancy as far as premature labor is concerned by the adequacy of the therapy during the 6th or 7th week. This is a very interesting point Progestational treatment during the early period may have an important influence on the full implantation and the further development or maturation of the placenta at this stage This may be a very critical phase of gestation

M L HOPWOOD Dr Bitman s paper entitled Estrogen Determination in Blood and Body Fluids of Cattle is now open for discussion

JACK GORSKI In view of Klynes (*Biochem J* 1956) isolation from cow urine of 17 α-estradiol which has low biological activity could some of your fluorescent fractions thus be accounted for?

JOEL BITMAN It is possible I do not recall the amount isolated If Mr Gorski has that information, I could better estimate whether that could be contributing to the fluorescence which we determined

JACK GORSKI I do not remember the exact amounts I think it was about 100 gamma/l It was less than estrone but it was a substantial amount and no estradiol 17 β was found or reported in that paper (*J Endocrinol* 14 33 1956)

JOEL BITMAN Such amounts of 17 α-estradiol could contribute to the urinary fluorescence we measured although fairly good agreement was found between fluorescence and biological activity of urine

H NOWAKOWSKI Have you examined the estrogenic content of the ejaculate and what are the results?

JOEL BITMAN No we have not done that The only other biological materials we have examined are bile and feces

A M SORENSEN Was recovery checked of known amounts of estrogens added to spayed heifers urine or blood?

JOEL BITMAN No we have not carried out recovery experiments with the blood or urine of spayed heifers However we have injected estrogens and assayed blood samples of normal nonpregnant cows Our results agree essentially with those that Dr Melampy and his group reported (Duncan *et al* 1955) We obtained recoveries of 0 90% and 1 93% one hour post injection and the Iowa State group reported a recovery of 1 45% one hour post injection

A M SORENSEN At what period of pregnancy were samples of blood and urine taken?

JOEL BITMAN All pregnancy blood and urine samples were obtained from cows 6–8 months pregnant

L T SAMUELS Were methods of Brown and Bauld tried in your studies?

JOEL BITMAN The method of Brown involving chromatography of the methyl ethers of the estrogens was not attempted The procedure we used is very similar to the one that Bauld has published Our method is a modification of the Braunsberg method (Syer and Braunsberg *J Endocrinol* 7 lx 1951) and is similar to the procedure reported by Haenni *et al* (*J Am Pharm Assoc* **42** 167 1953) Within the last year we have investigated the Bauld method but have found no advantages in this procedure when compared with our own In fact we do not get as good a recovery also we have observed considerable tailing with the estradiol fraction

R M MELAMPY We have had considerable experience using the Szego and Roberts dialysis procedure (*Endocrinol* **41** 322 1947) on cow blood Our assays have been conducted by using the 6 hour test on 21 day old female rats (Astwood E B *Endocrinol* **23** 25 1938) as well as ovariectomized 21 day old animals Our results have been inconsistent and similar to those reported by Dr Bitman today I noticed in two of his experiments he indicated the presence of estrogenic activity in the dialyzates of cow blood We observed the same thing occasionally I am wondering if Dr Bitman would care to comment as to why we obtain this positive test only at certain times Is it the physiologic condition of the cow at the time the blood sample is drawn?

JOEL BITMAN As shown in one of the slides we obtained the same results that Dr Melampy just reviewed in which we determined estrogenic activity in one sample while various other samples from the same cow and the same pregnancy yielded no biological activity This same cow showed biological activity in her next pregnancy Twelve other cows however showed no detectable activity I think it is an individual matter in that one particular cow may at times have a slightly higher level than most other pregnant cows and therefore sporadically we can detect activity As most people in this field have come to realize the bovine seems to have a lower hormone output than almost any other species studied The steroid and gonadotropic excretion is much lower than in the human We are faced with an animal which has a low level of hormone excretion and we have no explanation for the occasional activity except that sporadic individual samples may have enough activity to be detectable

IV GENERAL PROBLEMS OF REPRODUCTION

CONTROLLED ESTRUS IN CATTLE

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THE importance of controlling reproductive processes in mammals is increasing with applications of new control methods recently introduced Techniques are being designed to prevent conception as well as abortion, and to redate or initiate estrus in order that the conception date may be controlled In artificial insemination of farm animals control of breeding dates would increase the efficient use of time and semen as many animals could be bred during a short period of time In ova transfer work synchrony of donor and recipient animals is a must to ensure favorable results

This discussion will center around the ovarian steroid hormone progesterone as it is a natural substance controlling estrual cycles (reviewed by Dutt 1953) and has been most extensively used It can be mentioned here that Smith *et al* (1957) found lactogen as they used it to be ineffective in prolonging the estrus cycle in the bovine This is in contrast to the evidence already alluded to by Dr Nalbandov earlier on the program regarding a positive effect with ewes Progesterone has been applied in several ways to control the sex cycle To enumerate briefly they are bring to estrus anestrous sheep or cattle not showing cyclic heat controlling date of estrus in cycling cows and sheep as an adjuvant to gonodotropic treatment of cattle to suppress ovulation in the human being and estrus in the dog

In spite of the practical nature of the problem very little work has been reported since the report of Ulberg (1955)

In Tables I II III and IV are presented data on attempts to recycle cows in experiments conducted by the Minnesota group (Dziuk 1955 Donker 1952 Nichols 1957 and Graham 1952)

The result of administering subcutaneously varying amounts of progesterone in corn oil and in a saline suspension is listed in Table I The administrations began from the 13th to the 17th day of the cycle and were continued for from 1 to 12 days It will be noted that the interval between the last administration and the appearance of heat varied from 3 to 8 days While data are not extensive enough to warrant drawing conclusions it appears that where 50 mg were administered daily the saline suspension had a longer interval between the last treatment and the appearance of heat than was observed with

TABLE I

Recycling Cows with Progesterone

(Donker 1952)

Cow No	Day of cycle injection begin	Type of treatment	No days treated	Interval between treatment and heat
686	14	50 mg in corn oil daily	11	5
886	17	70 mg in corn oil daily	6	5
T57	16	50 mg in corn oil daily	1	3
355	16	50 mg saline susp daily	4	8
686	15	30 mg saline susp daily	6	5
T42	15	50 mg corn oil	7	5
708	16	50 mg corn oil	7	6
706	14	50 mg corn oil	8	6
680	14	50 mg corn oil	4	7
355	14	50 mg saline susp 4 days skipped 1 day then 50 mg in oil 3 days	8	7
705	15	50 mg every other day in saline susp	9	8
355	14	30 mg saline susp daily	7	6
T89	14	30 mg saline susp daily	12	6
T79	14	30 mg saline susp 5 days 50 mg in corn oil 5 days	10	5
T80	14	50 mg corn oil daily	5	4
T89	14	30 mg saline susp daily	10	5
T88	13	30 mg saline susp daily	10	5

the corn oil solution of the same amount When 30 mg in saline suspension were used there was a tendency to shorten this interval

In Table II are presented the results of attempts of recycling in which progesterone was dissolved in corn oil and administered subcutaneously The beginning of treatment varied from the 13th to the 17th day of the cycle The amount of progesterone from 40 to 60 mg/day and the duration of the treatment from 4 to 18 days The interval between the last treatment to the onset of heat varied from 3 to 9 days

It is to be noted that within the limits studied there is no indication of any relationship between the day of the cycle when treatment began the number of days of treatment and the interval between last treatment and the beginning of heat It was found that as dosage level increased there was a tendency for lengthening the interval between treatment and heat Eight cows in this series failed to show any signs of heat

In Table III are listed the results of 17 trials in which the daily dosage was kept constant but the beginning of treatment varied between the 15th and 18th days of the cycle and duration of treatment from 4 to 12 days It

TABLE II

Recycling Cows with Progesterone

(Dziuk 1955)

Cow No	Day of cycle injection began	Dose in mg/day	Days treated	Days to heat post treatment
E799	14	50	10	5
E799	15	50	9	4
E799	15	60	11	9
E798	14	50	8	4
E798	14	50	9	5
E797	13	50	11	4
E797	15	50	9	4
E797	15	60	14	8
E797	14	60	10	7
E800	14	50	9	5
E800	15	50	7	6
E800	15	50	6	6
T65	14	50	10	5
T53	15	50	10	3
T53	14	50	13	4
T54	15	50	4	3
77	15	40	7	4
T8	17	60	4	3
929	15	60	18	5
T141	15	60	16	6
T140	15	60	5	5
T140	17	60	4	4
E801	15	50	13	3

Eight cows failed to show heat following withdrawal of progesterone

will be noted that the interval between the last day of treatment and onset of heat varied from 4 to 7 days and that variations in the time of beginning of treatment and duration of the treatment were not related to the differences in the time of appearance of heat

In Table IV are given the results of trials on six cows where recycling was done three consecutive times It will be noted that there is no significant difference in the interval between treatment and onset of estrus with successive recycling Follicles were developed in all cases but in third recycling no corpus luteum was formed in four of the six cows treated

In general of the data presented in Tables I II and III it was observed that the older and fatter cows failed more often to return to heat within the expected time Also these animals more often showed a tendency to develop cystic follicles following withdrawal of progesterone and consequently show various degrees of nymphomania (Dziuk *et al* 1958)

Ovulation which was detected by rectal palpations in many instances was normally related to estrus in the great majority of cases but several anamalous

TABLE III

Synchronization of Estrus Cycle

(Nichols 1956)

Cow No	Day of cycle injection began	Dose in mg/day	No days injected	No of days between end of treat and estrus
T132	13	50	10	5
E893	11	50	7	5
E895	17	50	5	4
E896	18	50	4	4
T141	15	50	12	4
T141	*16*	*50*	9	*5*
E898	15	50	4	4
E902	14	50	5	4
E897	*16*	*50*	7	*5*
929	18	50	8	5
E801	15	50	9	4
1114	14	50	9	4
E907	15	50	7	4
E909	16	50	5	4
E905	15	50	7	7
E902	15	50	9	6
E911	15	50	5	4
Mean	15 1	50	7 17	4 59
Range	11–18	—	4–12	4–7
Standard deviation				±0 866

cycles were seen One animal ovulated while showing an intense and some what prolonged heat and displayed metorrhagia concurrent to standing heat

Two animals which had been recycled on two previous occasions came into heat and ovulated 8 and 9 days after cessation of progesterone and then came into heat again in 4 and 3 days later and again ovulated No corpus luteum developed following the first estrus but one was found on the ovary of each cow after the second ovulation

Nine of the animals were bred at first heat after treatment and of these 2 conceived The remaining 7 and 2 animals which were not bred at first service were bred at the second heat period or at later heats until they conceived Other animals were bred after several intervening estrus periods and it was concluded that fertility though adversely affected at first heat was not permanently affected

From a report by Graham (1952) it appeared that recycling at three con secutive cycles altered the formation or maintenance of the corpus luteum When cows were examined 5 days after estrus there were several animals after the second or third recycling in which a corpus luteum was not palpable on the ovary The results of possible ovulation and development of a normal corpus luteum are given in Table IV

TABLE IV

Determination of Normalcy of Estrous Cycle as a Result of Progesterone Treatment

(Graham 1952)

Cow No	Treatment of 50 mg progesterone in days	Interval from withdrawal to estrus	Rectal palpation of ovaries	
			Estrus	Five days post estrus
First Recycling of Estrous Cycle				
E 53	5	4	Follicle	C L
E 54	5	4	Follicle	C L
E 55	12	4	Follicle	C L
E 57	7	4	Follicle	C L
E 59	10	5	Follicle	C L
E-62	20	4	Follicle	No C L
Second Consecutive Recycling of Estrous Cycle				
E 53	4	3	Follicle	C L
E 54	4	3	Follicle	C L
E 55	4	4	Follicle	C L
E 57	4	3	Follicle	C L
E 59	3	4	Follicle	No C L
E-62	4	3	Follicle	C L
Third Consecutive Recycling of Estrous Cycle				
E 53	4	3	Follicle	No C L
E 54	4	4	Follicle	No C L
E 55	4	4	Follicle	C L
E-57	4	4	Follicle	C L
E-59	4	4	Follicle	No C L
E-62	4	4	Follicle	No C L

From the work reported to date it is evident that estrus in the cow as well as in other species can be controlled within certain limits Much remains to be done regarding the limits of control of the time of estrus and ovulation and in improving fertility at the first heat period after treatment The point of greatest concern with recycled dairy and beef cattle is the low conception rate at first estrus Willett (1950) reported 11 of 22 animals conceiving at first heat after treatment when using two inseminations spaced 24 hours apart Trimberger and Hansel (1955) had only 3 out of a group of 24 cows conceive to first service after cessation of progesterone and 15 5 2 and 1 on succeeding estrous periods Ulberg (1955) presented data which suggested that larger dosages decreased conception rates at the second estrous period after treatment

In this report, he also suggests that the adverse effect may be due mainly to embryonic mortality

In making application of a progesterone recycling technique with beef animals Mellor and Cole (1956) used a single large subcutaneous injection of crystalline drug suspended in a 0 3% starch suspension (560–1120 mg) They found that administration of 750 I U of pregnant mare serum (PMS) after the last progesterone treatment resulted in a higher incidence of estrus within a shorter interval than if it were not used In what appears to be their most successful treatment heifers weighing approximately 550 lb (14–16 months of age) were given 560 mg progesterone followed with PMS 15 days later Con ception to first service was 17%

Contrary to evidence in an early report that progesterone recycling did not adversely affect fertility of recycled ewes later reports have shown that progesterone recycling reduced fertility O Mary *et al* (1950) using 10 mg progesterone injected subcutaneously on a daily basis for 14 days beginning after the last ewe of the experimental group had shown estrus found the lamb ing rate of the treated equal to the control group They obtained 19 lambs from 20 treated ewes and 20 lambs from 19 control ewes The duration of both the breeding interval and the lambing period was shortened as a result of treatment

Robinson (1956) treated Merino ewes with 10 mg progesterone in oil daily for 16 days Half of the treated ewes were given PMS on the day after stopping progesterone and the other half of the treated animals received no PMS Results indicated that progesterone used alone reduced fertility from control levels but that the addition of PMS overcame the difficulty In 104 control ewes 58 lambed to give a 71% lamb crop There were 104 ewes in the pro gesterone alone group of which 53 ewes lambed for a 62% lamb crop The group of 107 ewes which received progesterone plus PMS yielded a lamb crop of 79% with 61 ewes lambing Davies (1957) however using the same treat ment as Robinson found that progesterone with PMS caused a decided lowering of fertility with Merino ewes Davies observed that 30 of 36 treated ewes returned to heat a second time after having been bred at first service after treatment while the nonreturn rate was expected to be about 60% The ewes used by Davies were observed to show heat much sooner on the average than Robinson s group (22 vs 40 hours) Davies hypothesized that in his ewes there was a dissynchrony between estrus and ovulation because of early heat

Attempts to bring anestrous ewes to fertile service have recently received the attention of Raeside and Lamond (1956) Progesterone alone PMS alone or a combination of both were used using 105 Romney ewes Proges terone in oil injected subcutaneously for 4 days at the rate of 10 mg/day did not cause anestrous ewes to come into estrus or to ovulate PMS used alone resulted in 60% of the ewes ovulating but no estrus was shown Pro gesterone with PMS caused 60% of the ewes to show estrus and all of them

ovulated The reproductive tracts of all animals were examined histologically It was ascertained that progesterone affected in decreasing order of the following genital organs or parts of them uterine lining cervix vagina and oviduct It would seem that detailed histological examination, such as presented by Raeside and Lamond or more detailed histochemical examination of uteri as exemplified by the report of Rosenbaum and Goolsby (1957) and such as exemplified by the work of Folley and his group which was reported on yesterday will give important clues to explain and possibly overcome the difficulties encountered after using progesterone to recycle cattle and sheep

A recent report from Germany (Burkl and Kellner 1956) indicates that the primary difficulty of infertility after recycling should not arise as a result of ovulating young ova Their study of the maturation process in rat ovaries indicated that ova examined in ovaries in all stages of the cycle displayed all stages of the maturation process but that only those at certain advanced stages in the presence of follicle stimulating hormone would go on to ovulate while those reaching this stage prior to follicle stimulating hormone release regressed and degenerated There still remains the question of the fertility status of old ova in gonadotropin treated animals where the ovulating substance is given late A discussion of this point is given by Young (1953) in which he stressed the idea that old eggs in terms of the time of union of gametes led to fewer fertilizations and more abnormal embryos

In attempts to produce fertile ova from superovulated cows progesterone pretreatment has been compared to no pretreatment by Nichols *et al* (1957) Daily subcutaneous injections of 50 mg progesterone in oil preceded the administration of gonadotropins starting at the 15th day of the cycle The effect of progesterone treatment was evaluated by comparing the incidence of early corpora lutea and incidence of estrus in groups with and without progesterone Forty animals received progesterone and 26 served as controls Upon autopsy *of 36 animals 36% of treated animals and 82% of controls showed early* corpora Of the 40 progesterone treated animals 53% showed estrus after superovulation in contrast to 15% of the 26 control animals With but one or possibly two exceptions the animals showing estrus did not possess early corpora lutea In the cows producing fertilized ova 70% displayed heat with none of these animals showing an early corpus luteum No fertilized ova were recovered from 13 cows possessing an early corpus luteum Progesterone appeared to decrease the number of ova developed from gonadotropic treatment It should be clearly understood that the differences noted by Nichols *et al* (1957) do not satisfy the criterion of statistical significance but they do nevertheless appear to indicate real differences and would probably have been significant had greater numbers of animals been employed

Progesterone has been compared to several progesterone like compounds in recent years Zarrow *et al* (1957) compared the activities of seventeen such compounds by comparing their activities using the Hooker–Forbes test They

pointed out important species differences in the reactivity to the compounds tested 17 hydroxy progesterone was 60 times as potent as progesterone by their assay using the mouse, but was not active in causing progestational activity in the human

From the work reported to date it is evident that estrus in the cow, as well as in other species can be controlled within certain limits Much remains to be done regarding the limits of control of the time of estrus and ovulation and in improving fertility at the first heat period after treatment

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PROBLEMS OF INFERTILITY IN THE DAIRY HERD

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It is the purpose of this paper to discuss some of the factors other than contagious disease which may alter reproduction in the dairy cow Emphasis will be placed on individual cow problems many of which are very familiar to the veterinarian but which often respond unsatisfactorily to therapy and also on minor factors which are important causes of infertility but may be overlooked because of inadequate breeding records or lack of persistent observation Much of the following data will be characteristic of the extra well cared for herd and may not necessarily apply to the average herd Most of these problems need further study before they are fully understood and an attempt will be made to emphasize these needs in the hope that it will stimulate further discussion and continued research by members of this group

THE APPARENTLY NORMAL COW

The main problem facing the dairyman today aside from contagious genital disease is the cow which appears grossly normal in every respect yet fails to conceive after repeated breedings to known viable semen Diagnosing the cause of infertility in these cases presents quite a problem and many of them are diagnosed and corrected only after a method of trial and error This of course is a long and costly procedure and many cases are disposed of before a correct diagnosis is made Obviously the practitioner needs help on this syndrome and research should be continued in this direction

Research to date indicates that a high percentage of cows that are having regular heat periods and appear grossly normal otherwise are failing to reproduce because of a mild uterine infection of non specific origin Fitch and Bishop (1932) have found that the healthy bovine uterus is in general free of bacteria De Camp (1935) has reported that metritis is most commonly caused by *Streptococci Staphylococci Corynebacterium* and *E coli* all of which are found in the normal surroundings of the dairy herd Easley *et al* (1950) found that 75% of the repeat breeding cows in a generalized survey had a low grade infection of the uterus

INTRA UTERINE THERAPY AT BREEDING TIME

These findings have stimulated the use of various intra uterine treatments some of which have met with considerable success Iodine and oil weak Lugol s solution and Sulfamerazine and oil were among the drugs first used for this purpose and the injections were made once or twice a week throughout one or more complete estrous cycles with the last treatment at least 4 days before anticipated heat It was even suggested that the anestrous phase was the optimum time for treatment because the uterus was somewhat flaccid at this stage and would therefore retain the medication longer Results from this routine were only fair even after the antibiotics came into common usage In the meantime the bovine uterus was found to be more susceptible to infection during the luteal phase or when progesterone predominates and conversely the uterus appears to resist infection during the heat period or when estrogens predominate This information explains in part the inconsistent results obtained from promiscuous intra uterine therapy and it also opened the way for the current practice of treating the uterus when estrogens dominate the cycle

Van Demark *et al* (1952) found that spermatozoa deposited in the cervix by artificial insemination will reach the ovarian portion of the oviducts in as little as 2 5 minutes Similar rapid transport takes place after natural breeding and if conception occurs it takes at least 4 days for the embryo to reach the uterus from the oviducts With these facts in mind it appeared practical to treat the uterus at any time from 10 minutes to 3 days following breeding and a trial was set up on this basis Combiotic* (penicillin–streptomycin) was the drug of choice because of its low toxicity to spermatozoa and its broad spectrum activity against the organisms commonly found in the bovine uterus

It was decided that 100 cows would give an accurate evaluation of this procedure but before the records were tabulated 130 cows had been treated *Sixty five cows or 50% of the group conceived from the breeding at the time* of treatment and 31 cows or 24% of the group conceived at the next breeding without any further treatment This made a total of 74% conception following one treatment with Combiotic and these cows were bred from one to 13 times with an average of 3 3 breedings prior to treatment Twenty four cows or 18% conceived after additional therapy with Combiotic and/or other therapy and *11 cows or 8% of this group of problem cows were finally disposed of* as sterile

Cows failing to respond to one or two treatments with Combiotic usually would not respond to numerous treatments and would eventually fall into one of several categories First a few of them would conceive without any further treatment if bred long enough Also a few of them are ovulating too late and may respond to a follicle stimulating hormone Fourteen cows or 25 5% of 58 cases in one trial conceived following the intravenous use of Gonadogen

* Pfizer Laboratories

early in the heat period although they had been bred an average of 6 6 times before Gonadogen therapy Another group of cows will continue to have normal heat periods for a while and then develop cystic degeneration of the ovaries This would suggest that a hypo functioning pituitary gland is pro ducing just enough gonadotropins to stimulate a heat period but not quite enough to round out a completely normal cycle in all its phases because many of these cows will exhibit a normal heat period and conceive shortly after they are treated for cystic ovaries

Observations have disclosed also that a few of these apparently normal cows may be harbouring a uterine infection that is not readily diagnosable by rectal examination nor by the character of the vaginal mucus at estrum and it is a type of infection that responds poorly to Combiotic therapy It takes the action of several heat periods or supplemental estrogen therapy to expel a rather small amount of thick heavy pus from the uterus and allow the endo metrium to resume its normal function after which conception usually occurs

Intra uterine therapy at breeding time has continued to give good results in selected cases verifying the fact that low grade uterine infections are an im portant factor in delayed conception These findings prompted another trial on intra uterine medication this time immediately following parturition

INTRA UTERINE THERAPY AT PARTURITION

One assumption is that most of these mixed uterine infections are initiated at parturition time or during the puerperal phase *post partum* and not after *the uterus has returned to its non gravid state If this assumption is correct* appropriate treatment of the uterus as soon as the placenta is passed would probably prevent these chronic infections and increase subsequent breeding efficiency Therefore every female that calved over a period of about 18 months was placed consecutively in one of three groups Group 1 consisted of the untreated controls group 2 was treated with two 1g tablets of Terramycin and group 3 was treated with two uterine tablets composed of Neomycin urea boric acid and sulfathiazole It was originally planned to have at least 100 cows in each group however before this goal was reached it became obvious that the controls or the untreated group were breeding better than either treated group and the trial was discontinued The following table summarizes the results of this trial and definitely shows that breeding

	Group 1 No treat ment	Group 2 Terramy cin	Group 3 Neo-mycin
Number of cows in trial	79	75	74
Number conceived	73	67	71
Conceived at first service	44 (60%)	33 (49 %)	29 (40)
Conceived on first 3 breedings	85 3	73 3 %	72 9 %
Breedings per conception	1 67	2 24	2 56

efficiency is not enhanced by routine medication of the uterus immediately *post partum* but to the contrary conception is actually delayed by this practice

Hanold (1957) did a group comparison between the breedings per conception for Group 1, no treatment and those for Group 2 treated with Terra mycin, according to the method presented by Snedecor (1953) for two groups of different sizes and found the difference to be statistically significant A separate group comparison was done between Group 1 no treatment and Group 3 treated with Neomycin and the difference was highly significant It is difficult to understand why medication of the uterus at parturition time in the normal cow should lower subsequent breeding efficiency especially when intra uterine treatment is so successful when used at or near the heat period One can only assume that the antibiotics destroy beneficial organisms enzymes or other factors which are necessary for the normal cleanup of the uterus following parturition This of course does not infer that antibiotics or other therapy should not be used following pathological parturition to prevent septicemia

ENDOCRINE DISORDERS

Endocrinologists and others working in the field of endocrinology have compiled considerable information on ovarian abnormalities in the dairy cow and advancements have been made in the diagnosis and treatment of these conditions Clinically speaking however diagnosis and treatment of endo crine disorders is still a major problem even for the specialist and the com plexity of the problem is not fully appreciated until accurate records are re viewed on a large number of cases Such a review was made by Erb and Morrison (1954) from the breeding records of a large herd of registered Holstein cattle covering a thirty year period from 1920 to 1950 Most of this data was compiled before gonadotropins were in common usage and manual manipulation of the ovaries was the main therapy

The incidence of ovarian abnormalities and the effect of these abnormalities on the fertility of this herd both directly and indirectly are tabulated in the following table

	Cystic ovaries	Short heat periods	Herd average
Incidence	12 13%	17 27%	—
Breedings per conception	3 28	3 25	2 12
Abortions	10 8%	9 2%	6 8%
Twinning rate	9 3%	7 7%	4 73%

This gross summary suggests that cystic ovaries and short estrous cycles directly delayed conception in 29% of the cows in this herd at some time

during their lifetime and indirectly by increasing the abortion rate and twinning rate above herd average The twinning rate was largely governed by the time of conception and the method of therapy following cystic ovaries Unexpressed cysts resulted in a twinning rate of 4 3% which was essentially herd average whereas expressed cysts were accompanied by an incidence of 11 3% Those conceiving within 25 days had a twinning rate of 23 3% while cows conceiving within the first 75 days twinned at a rate of 15 7% Cows conceiving later than this conceived at a rate similar to herd average

The increase in abortion rates following cystic ovary cases is hard to evaluate but probably is associated with the increased twinning caused by cystic ovaries and possibly from uterine infection which is incriminated as one of the causes for cystic ovaries The records from this herd have not been summarized as yet in this respect but it appears that far more cases of twins are aborted than ever reach maturity and that for every case of visible abortion there are a number of cases that have been passed unrecognized in the embryonal stage Add these factors to the decrease in breeding efficiency due to retained placenta following twin births or abortions and cystic ovaries are indirectly responsible for more infertility than first meets the eye Also cows with a cystic history tend to show recurring cysts at a rate three to four times that of first offenders and the cystic ovary syndrome is definitely an inheritable character

Environment such as forced feeding and high conditioning for optimum production plus the delay in breeding that is necessary for the cow to produce a 365 day record is a management practice which percentagewise is responsible for the majority of cystic ovaries in this herd This is under standable when one realizes that the endocrine system of a high producing dairy cow remains in a rather delicate balance during heavy lactation and the gonadotrophic portion of the anterior pituitary gland may become hypo functional to the extent that the gonads fail to receive the proper stimulus to initiate or complete a normal estrous cycle Generally speaking each successive heat period up to 100 days aids in preparing the uterus for conception how ever each estrous cycle beyond this arbitrary point increases the chance for endocrine dysfunction and an arrest of the cycle in one of its phases

Another phenomenon associated with cystic ovaries is that of post concep tion estrus Erb and Morrison (1956) found that 5 6% of all the cows studied in their herd survey showed one or more heat periods after they were pregnant and that 71 3% of them had been cystic before conception This tendency is quite pronounced in one cow family and repeated pregnancy examinations are necessary to make sure that these cows are still safe in calf This phenomenon is of practical importance because pregnant cows may be thought to be sterile and sent to slaughter and also because of questionable paternity when *different bulls were used*

DIAGNOSIS OF CYSTIC OVARIES

Cystic ovaries are usually thought of as either follicular or luteal in nature depending upon their physical appearance and the way in which they influence the estrous cycle However, many cystic ovaries are not typically follicular or luteal in appearance nor in the way in which they influence the estrous cycle Many of these cysts show the physical characteristics of a follicular cyst that is thin walled and easily ruptured yet they are no larger than a normal Graffian follicle and they cause only a very mild polyestrus that may be easily missed if not carefully observed Others may be confused with a normal embedded yellow body yet when they are ruptured there is no evidence of luteal tissue present and the main clinical symptom is that of anestrus This type of cyst is plentiful in the cow that has been fresh 4–10 weeks and has not yet had a normal heat period and also in heifers Some of these cysts will disappear spontaneously before time to breed the cow but others will persist for long periods unless they are treated Clinical symptoms are a slightly swollen vulva and a persistent watery discharge of mucus from the vagina Physically both ovaries appear about the same size and can be very easily confused with a normal ovary except there is a tenseness about one or both ovaries that can be recognized with experience A quick firm thrust with the thumb while the ovary is held securely in the hand will usually rupture the cyst leaving practically no substance left to the ovary If a yellow body should be present this procedure will partially dislodge it producing a crunchy or crepitating sensation again recognized by experience To avoid injury to the ovary just the right amount of pressure must be used Once these cysts are ruptured the cow will usually proceed with a normal estrous cycle without any further treatment

Typical follicular cysts which cause polyestrus present no diagnostic problem nor does the typical luteal cyst which is responsible for some cases of anestrum therefore they will not be discussed at this time

RESPONSE TO CYSTIC OVARY THERAPY

Albrechtsen (1917) as far back as 1910 maintained that cystic ovaries were caused by metritis and cervicitis and that he had excellent results in curing cystic ovaries as well as the metritis and cervicitis merely by treating the uterus and cervix with a strong iodine solution Today the use of gonadotropins is the preferred treatment but unlike Albrechtsen s experience in 1910, results of today are far from excellent and there is a lot yet to be learned In order to learn more about cystic ovary therapy a summary was made on 113 cows in one group that had received either chorionic gonadotropin or anterior pituitary gonadotropin one or more times as a treatment for cystic ovaries In general the manufacturers recommendations were followed very carefully Most of the cysts were ruptured manually or drained with a needle to supplement this treatment

Thirty seven cows or 32 5% failed to conceive following therapy and they were treated an average of three times with several cows being treated eight times Thirteen of these 37 cows resumed what appeared to be normal heat periods for a while only to become cystic again Seventy six cows or 67 5% conceived with an average of 1 67 treatments 48 of which conceived after one treatment The remainder of the group took up to six treatments and as long as 667 days before conception occurred making an over all average of 121 days from initial therapy to conception Another group involving less cows, was treated with a specific brand of chorionic gonadotropin for clinical evaluation purposes Sixty per cent of the cows in this trial responded to therapy in a reasonable manner and a total of 82% became pregnant eventu ally however the time from initial therapy to conception averaged 103 days The time was extensive on these trials partly because it was the custom to breed the cow at the second or third heat period following gonadotropin therapy to avoid multiple births Erb s work has since proven that multiple births are due to the cystic ovary and not necessarily to the gonadotropin Results are better if the cow is bred at the first heat after therapy before she has had a chance to become cystic again Anaphylactic reactions occurred in four cases following the use of chorionic gonadotropin and in several cases of manual rupture without hormone therapy There were no reactions in those cows treated with Vetrophin* (anterior pituitary gonadotropin)

More recent experience with this problem suggests that treatment can become too vigorous and that many cases are retreated before they have had time to establish a physiological endocrine balance thus delaying a normal estrous period Also repeated treatment especially in the form of manual rupture increases the destruction of ovarian tissue and may cause inflamma tion of the ovaries with subsequent adhesions and permanent sterility If treatment has to be repeated the cyst should be drained with a hypodermic needle via the vagina

The draining of a cystic ovary with a hypodermic needle brings up one of the more important advances in cystic ovary therapy This procedure has several advantages over the conventional practice of manual rupture First less damage is done to the ovary if aseptic precautions are followed and it also eliminates the follicular fluid from the body It is a personal assumption that the rupture of a cyst into the abdominal cavity actually injects the animal with the same non physiological amount of hormone that is already altering the estrus cycle In other words if the estrogen level of a follicular cyst is high rupture of this cyst into the abdominal cavity will allow rapid reabsorption back into the blood stream and cause polyestrus all over again and the same goes for a luteal cyst anestrus may continue longer if the contents of the cyst are allowed to be reabsorbed Records have not been summarized in this respect but results from cystic ovary therapy appear superior when this technique is used

* Abbott Laboratories

This technique may also open up new channels for additional research on ovarian secretions because it is possible to collect ovarian fluid for analysis and if a simple test could be developed to determine the hormonal content of such a cyst it might be possible to use a dosage of the proper gonadotropin that would be physiological for each individual

SUMMARY AND CONCLUSIONS

Some of the infertility problems of the well cared for dairy herd have been discussed in detail Data herein presented was gathered partly from specific trials that have been conducted within this herd and partly from a review of the breeding records over a thirty year period

Research to date indicates that a high percentage of the grossly normal cows are failing to conceive because of a low grade uterine infection In one trial, 74% of 130 cows conceived following one intra uterine treatment with Combiotic given any time from ten minutes to three days after breeding and these cows had been bred previously from one to thirteen times with an aver age of 3 3 breedings Twenty four cows or 18% of the group conceived after additional therapy Some were ovulating too late and responded to a follicle stimulating hormone and some developed cystic ovaries and conceived after a luteinizing hormone was used Eleven cows or 8% of the group were classed as sterile

The intra uterine use of Combiotic at breeding time has continued to give good results but routine use of other antibiotics in the uterus at parturition time as soon as the placenta is passed did not increase subsequent breeding efficiency as was assumed but to the contrary it actually increased the num ber of breedings necessary for conception in the treated groups

Considerable time had been devoted to the cystic ovary syndrome because it is felt that this condition is more serious than is generally assumed especially in certain herds and this is one field where continued research would be very fruitful A thirty year summary on one herd disclosed a cystic incidence of 12 13% and a 17 27% incidence of short estrous cycles which according to this survey is practically as important as cystic ovaries in causing infertility Gonadotropins are useful in treating cystic degeneration however 32 5% of 113 cows in one trial failed to conceive after an average of three treatments Of the 67 5% that did conceive only 63% conceived with one treatment (42 5% of the entire group treated) and the others were treated up to six times and it took 667 days before conception occurred in one cow These problem cases made an average time of over 100 days from initial therapy to conception in the entire group and this delay in breeding plus the cost of therapy would be prohibitive in the average commercial herd The time was extensive in this trial partly because it was the custom to delay breeding until the second or third heat period to avoid multiple births It has since been proven that multiple births are caused by the cystic degeneration and not

necessarily by the gonadotropin and this time is reduced by breeding the cow at first good heat period following therapy before she has had a chance to relapse

The fact that a fair percentage of the cases that do respond to gonadotropic therapy do so at one treatment would suggest that more cases should respond if it were possible to give a physiological dose of the proper hormones to each individual Improved methods of diagnosis possibly by chemical assay of the blood or ovarian secretions may some day make this possible

This discussion of infertility in the dairy cow has by no means been com plete However a big majority of all individual cases will fall within the realm of this discussion The remaining cases may be caused by minor endocrine disturbances not discussed poor management heredity and anatomical variations in the genitalia With our present knowledge of the subject all cases of infertility cannot be corrected even when constant supervision of a herd is possible However infertility work is most successful when each herd can be observed at regular intervals and treatment prescribed at a time when it is most effective

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LOW-LEVEL ANTIBIOTIC INFUSIONS IN BOVINE REPEAT BREEDERS

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INTRODUCTION

AN EXTENSIVE review of the evaluation of uterine infusions for the treatment of bovine infertility amenable to this type of therapy has been published by Roberts (1956) who also reported his experience with various agents from 1944 to 1953 Usually problem animals were infused when not in heat If a repeat breeder was treated at the time of estrum she was not usually inseminated at that time apparently because it was felt that semen viability would be impaired by the type of infusion employed or perhaps because of extensive conception impairing uterine pathology

In addition to repeat breeders showing obvious evidence of endometritis or other genital complications there are many which do not conceive but for which there is no apparent reason for continuous failure In recent years the terms low grade or non specific metritis have been more frequently employed to signify a condition of the endometrium due to unrecognized bacterial populations which would apparently preclude conception or at least lower fertility presumably by creating an unfavorable environment for the fertilized ovum Even more recently it has been theorized that mild undiagnosable metritis might be in part responsible for early embryonic death or for the degeneration and subsequent absorption of the embryo The present investigation deals mainly with cases in this category

It was felt that in repeat breeders showing no gross pathology the application of a low level antibiotic infusion might overcome unfavourable uterine conditions and permit pregnancy to occur without loss of a cycle

MATERIALS AND METHODS

Among substances tested for compatibility with sperm one was chosen because of its broad antimicrobial spectrum and because solutions in sterile distilled water have a pH range from 6 3 to 6 7 This substance is a calcium–

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oxytetracycline*-glucose complex The amount used for each treatment was equivalent to 100 mg of oxytetracycline bio activity The volume of solution was most often 10 m³ but ranged from 5 m³ to 20 m³ Infusions were adminis tered by means of a 10 m³ syringe through the same plastic cannula used for the insemination No special adapter was found necessary as a small wad of cotton impaled on the needle seemed to furnish a satisfactory seal

The antibiotic infusion was applied to dairy herds in Indiana New York Pennsylvania and Illinois In the Indiana trial there were 4 untreated controls and eight to which the infusion was administered within seconds after insemination In the New York trial there was a single group of 94 repeat breeders which served as their own controls being compared on a basis of their conception records before and after the infusion program These animals also were treated within seconds after artificial insemination One group studied in the Pennsylvania trial comprised 18 repeat breeders with no evidence of pathology The second group comprised nineteen repeat breeders in which there was irregular heat metritis or enlarged cervix Infusions were administered two to 24 hours post insemination The comparison in this trial also was on a basis of conception history before and after the douching program

Dr W G Huber Cissna Park and Dr R P Link of the University of Illinois conducted the trials in that state Their complete results will be reported elsewhere but they have kindly permitted mention of their initial data from two groups One of these numbering 24 repeat breeders without obvious pathology received the antibiotic infusion 24–72 hours post insemination The other group numbered 28 animals for which pre treatment breeding records were not available but which were affected in various degrees by acute or chronic metritis and/or cervicitis These cows were given the antibiotic infusion 30 to 45 days *post partum* and insemination was per formed the first or second estrum after medication For both groups compari sons of conception rates before and after infusion were made

When the infusion was used immediately after artificial insemination the semen was deposited with routine equipment as high as possible in the uterine cavity The insemination cannula was then withdrawn a few centimeters and the infusion placed at the cervical region of the uterus No effort was made to distribute the medication as it was determined from study of the uterine mucosa of a cow killed twelve hours after infusion that shortly after administration the antibiotic was uniformly well dispersed within the uterine body and the two horns When using the infusion at any time during the 24 hours preceding or the 24 hours following insemination or during the so called breeding rest the solution was deposited with the same equipment and the same technique as any other uterine medication would be applied

* Trade mark of Chas Pfizer & Co Inc for oxytetracycline is Terramycin

RESULTS

The results are presented in Table I It will be observed that in the Indiana trials all of the animals to which the infusion was administered within seconds after insemination conceived with eleven services or 1 37 per animal Of the four control animals after thirteen services or 3 25 *per animal only one conceived*

In the New York trial the previous breeding records showed that 327 ser vices had been given unsuccessfully to the group of 94 an average of 3 44 services per cow After infusion a 66% conception rate was obtained with a total of 94 services An effort was made in this trial to exclude from this experiment any animals with apparent anatomic abnormalities obvious irregularity of cycle or other physiological dysfunction

In the Pennsylvania trials the group of 19 repeat breeders without evidence of pathology had received prior to infusion 3 37 services per animal without occurrence of conception in any After infusion a single service per animal resulted in pregnancy in 78 9% The group of 18 with irregular heat enlarged cervix or metritis had achieved no pregnancies prior to infusion although 3 44 services had been given per animal Subsequent to infusion a single service per animal resulted in a conception rate of 50% In the Illinois trials among the repeat breeders without obvious evidence of pathology there had been 3 21 inseminations per animal prior to infusion but without success After infusion all animals conceived after 1 24 services per animal Among the 28 with pathology of the cervix or uterus the breeding record prior to infusion was not known at the time of this report All animals became pregnant despite the existence of pathology with 1 21 services per cow after infusion 30 to 45 days post partum

The statistical method of Chi square for attribute data (Snedecor 1956) was applied to evaluate significance These calculations showed that in all com parisons the improvement in conception rate following antibiotic infusion was significant to a degree of 95% or greater on the basis of the number of animals per group Such calculations could not be applied to the Illinois group for which previous reproductive performance had not been recorded

DISCUSSION AND SUMMARY

Infusions of relatively small volume prepared by dissolving in sterile neutral distilled water an amount of calcium–oxytetracycline–glucose complex having a bio activity of 100 mg of oxytetracycline hydrochloride were applied at or near insemination or during breeding rest to known repeat breeders Significantly higher rates of conception occurred in all groups following the use of the antibiotic infusion *In some groups there was 100% conception from* approximately 1 2 services per animal following the use of this infusion whereas there had been no conception before treatment from a larger number of services per animal

TABLE I

L L A Infusions—Effect on Fertility of Dairy Cows

Group	No of cows	Pathology	Breeding record before infusion program	Record after L L A infusion	Vol of infusion	Administration time as related to insemination
Indiana control	4	Neg	8/4 2 00 0%	13/4 3 25 25 %	None	
Indiana treated	8	Neg	16/8 2 00 0%	11/8 1 37 100 %*	10 cm³	Immed post insem.
New York	94	Neg	323/94 3 44 0%	94/94 1 00 66 %*	10 cm	Immed post insem
Illinois	24	Neg	77/24 3 21 0%	30/24 1 24 100% *	20 cm³	24–72 hours post insem.
Illinois	28	Pos (a)	Not available	35/28 1 21 100 %	20 cm³	30–45 days *post partum*
Pennsylvania	19	Neg	64/19 3 37 0%	19/19 1 00 78 9%*	5 cm³	2-24 hours post insem
Pennsylvania	18	Pos (b)	62/18 3 44 0%	18/18 1 00 50%*	5 cm³	2–24 hours post insem.

In fourth column the fraction shows in the numerator number of services in the denominator number of animals served Between commas is the average number of services per cow Last figure is percentage of animals conceiving

(a) Acute or chronic non specific metritis and/or cervicitis

(b) Irregularity of cycle metritis enlarged cervix

* Significant over pre douche period 95 % confidence or greater

In the New York trial with 94 cows, the 66% conception rate obtained with one service per animal when the infusion was given a few seconds post insemination shows that the antibiotic, applied according to the pro cedure outlined is compatible with semen and uterine physiology of estrum and may be used within seconds after insemination However, in the Illinois and Pennsylvania trials, in which the infusion was administered after a lapse of time there was a higher conception rate indicating that a delay of hours or possibly minutes between insemination and infusion may be desirable

The antiobiotic infusion also appears to be an aid to conception by cows with acute or chronic nonspecific metritis and/or cervicitis This is shown by the Illinois group with unrecorded pre douche breeding records These all conceived with an average of 1 21 services after infusion during breeding rest

It is suggested that the higher percentages of successful nidation achieved following the infusion was probably because of suppression of existing although undiagnosable bacterial populations on or within the endometrium of the bovine repeat breeder

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THE PRESENT STATUS OF THERAPY IN ANIMAL INFERTILITY

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STATISTICS show that a cow produces an average of 3 5 fecundated ovules which will produce 2 5 living calves although we know there are about 80 000 possible ovules in the ovary of a heifer These alarming figures quoted by Philodore Choquette (1956) at the recent annual meeting of the Canadian Veterinary Medical Association points out the striking wastage in animal reproduction There are many factors which contribute to this situation some of which yield to therapy but many of which resist tenaciously all attempts to correct the cause Needless to say infertility in animals is a vitally important issue in the economy of the human race

In developing the theme of this paper it is necessary to present information relative to therapy resulting from experiences in the treatment of sub-fertile animals which have been accumulated and reported incident to the practice of veterinary medicine One must concede immediately that there are some patterns of treatment the validity of which cannot be proven conclusively by the standards which are set up for formal research Some styles of therapy do not lend themselves to controlled experimental procedures either by virtue of the fact that it is impossible to set up valid controls because of the physio logical variables involved or the economics of the situation at hand precludes such a well organized program The purpose of this review is to record the present status of therapy in animal infertility based on both exacting scientific data and reliable clinical reports

Specific reproductive diseases (such as trichomoniasis vibriosis brucel losis viral catarrhal vaginitis coital exanthema contagious granular vul vovaginitis) *and the nonspecific bacterial infections incident to parturition* are usually a herd problem and can be handled with a satisfactory degree of suc cess by what we should refer to as therapeutic management and the use of antibiotics sulphonamides and/or specific drugs known to be effective against the specific disease Modification of herd management alone will often improve breeding efficiency particularly in herds using an artificial breeding program

The more troublesome infertility problems lie within the realm of nutrition physiology and endocrinology thus these phases will be discussed in this paper

The incidence of infertility in male animals, particularly in the bovine species has become more obvious to us through the years of expanding artificial breeding services To date however, the treatment of these animals has been disappointing Except for sporadic reports in the literature from research workers and veterinarians there are no repeatable successful treatment schedules for sterile or sub fertile males

Over the past five years we have had the opportunity to study in detail 10 sub fertile bulls of both the dairy and beef breeds, presented with sufficiently accurate histories and reliable data to justify critical research as to cause and possible correction (Gassner 1955) These bulls exhibited fairly normal sex drive but produced abnormal semen showing either oligospermia or azoospermia Treatments with various commercial gonadotrophin preparations steroids thyro active agents and combinations thereof for intermittent periods ranging from 2 to 12 months yielded discouraging results Of this group 3 bulls were returned to service as breeding bulls The remaining 7 did not respond although 1 animal who had never sired a calf was maintained at the research station for more than 2 years The three that were returned to service were treated with several series of special gonadotrophin preparations one containing follicle stimulating fraction and another luteinizing fraction given in various combinations and supplemented with testosterone

Darlington and Chassel (1956) reported the use of tocopherol succinate to increase libido and stimulate sperm production in the stallion Oral administration of 1000 units of this compound daily during the breeding season has resulted in marked improvement in sex drive and capacity to cover mares quickly as well as increased sperm concentration and motility in a quarter horse stallion under care of the author Withdrawal of the medicine causes recession of sexual activity within a week to 10 days

The effectiveness of testosterone or other androgens on spermatogenesis when given alone is so variable that they cannot be classed as a successful therapeutic agent for conditions other than decreased sex drive

Recently the use of Prednisolone (δ 1 hydrocortisone) on arthritic bulls has brought to light an increased sperm concentration and lends support to the work of Cupps *et al* (1955) who have indicated that the adrenal cortex is sometimes involved in sterility of cattle

Therapy in the future may consist of various combinations of steroids and gonadotrophins and even corticoids (Rasbech 1953) as more information becomes available to the clinician through research

Many beef bulls presented to the sterility clinic are valuable show stock carrying excessive flesh put on during a lifetime of supra natural feeding conditions Thyroid therapy stimulation either through injections of thyroxin or by feeding thyroprotein (iodinated casein) is as effective a treatment as any providing that the obesity has something to do with the lowered fertility (Schultze and Davis 1946 Maqsood 1951 Rasbech, 1953)

Exercise is certainly indicated if only from the standpoint of improving general health and hardening of the bull for the physical demand during the breeding season It is true that little or no effect on semen quality has been demonstrated under carefully planned experiments (Lepard *et al* 1941 Snyder and Ralston 1955 VanDemark *et al* 1956) but many workers have indicated that exercise is necessary for certain bulls (Woodward 1920 Bartlett and Perry, 1939 Hamilton and Symington 1939 Hander 1955)

It is evident from results accumulated by veterinarians working on the Colorado Bull Evaluation program (Faulkner and Hill 1956 1957) that testic ular hypoplasia may be of greater incidence in range bulls than heretofore suspected Response to treatment or spontaneous recovery is dependent upon the degree of degeneration and the basic cause Improvement rarely occurs over a period of from 6 months to a year

It may be said currently relative to the treatment of the infertile male that no single therapy nor complex pattern of therapy is truly effective In spite of the spontaneous recovery of some males subjected to rather empirical treat ment we need considerable more information on basic physiology of repro duction before diagnostic procedures can be developed which will point the way to successful treatment

The role of nutrition still presents a confusing picture It is well established that most nutritional problems involve multiple deficiencies (Asdell 1949) and for the most part clinical symptoms other than reproductive inefficiency become apparent first Animals are most vulnerable to nutritional deficiency effects during the growth period (Asdell 1949 Hammond 1952 Reid 1957) Hammond (1952) propounds that young animals on a high plain of nutrition develop target organ sensibility earlier so that the various organs respond to hormones earlier in life

The clinician is often required to decide whether or not a given male of a certain species or breed found to be sub fertile or reproductively inefficient is yet sexually mature Experiences in evaluating semen of great numbers of young bulls presented at random indicates that bulls mature over a consider able range of ages (Faulkner and Hill 1956 1957) Some possess sexual desire and physical attributes as well as mature semen as early as 11 or 12 months of age while others may not reach sexual maturity until 24 to 28 months of age It is questionable whether one can say that a bull is sexually mature and ready to serve a normal sized harem without a critical evaluation of the entire reproductive system including several semen samples Perhaps some of the infertility in later life results from overwork because of the assump tion on our part that if a bull is a year old he must fall within the sexually mature class His plane of nutrition during development may well account for this tardy maturity

Phosphorus deficiency has definitely been incriminated in the sterility picture (Asdell 1949 Hignett and Hignett 1951 Hignett 1956) usually

manifested by anestrous delayed heat periods an above average incidence of so called silent heats and prolonged interval between calving and the first estrous period Hammond (1952) suggests that phosphate deficiency inhibits the production of pituitary FSH and the situation can be brought about by low level phosphorus intake in the ration or by a deficiency in the soils upon which the feeds are grown, or through the effects of heavy lactation in spite of a seemingly plentiful supply Blood serum levels of 2–8 mg/100 cm³ are con sidered normal for most domestic animals (Dukes 1955) Cattle should re ceive about 15 g of phosphorus per day plus an additional 3 g for lactating cows (Ldds *et al* 1952) Hignett of England (1956) recommends 50 g plus 20 g for each gallon milk daily

The ratio of dietary calcium to phosphorus influences the reproductive efficiency of cattle Hignett (1951 1956) states that where the calcium (Ca) exceeds the phosphorus (P 205) animals conceive most readily The optimum ration approaches 1 5–1 Normal blood serum values for calcium in animals range between 9 and 15 mg per 100 cm³ (Dukes 1955)

Bentley and Phillips (1951) have pointed out that a ration containing less than 20 p p m of manganese might affect heat periods and conception rates

Other trace minerals such as iron cobalt iodine etc may exert hidden effects thus warranting therapeutic use of trace minerals in certain herd problems

Avitaminosis A apparently does not cause distortion of heat cycles although the number of services required for conception may be more than average Weak calves calves born dead and retained membranes are common symptoms In most instances symptoms of weakness night blindness rough hair coat anorexia and even prostration occur in mature males before semen quality and fertilizing capacity are impaired Young males are more vulner able to vitamin A deficiency and may mature later than normal for the breed, or fail to serve females shortly after first sexual activity Semen from such bulls is higher than normal pH (normal reaches about 6 7) and does not store well (Sutton *et al* 1940 Davis and Madsen 1941 Thorp *et al* 1942 Erb *et al* 1944 Hodgson *et al* 1946 Madsen *et al* 1948 Asdell 1949)

Vitamin A given at the rate of 3000 I U /100 lb body weight until serum values equal 20–100 μg/100 cm³ should be an adequate supply A therapeutic level of carotene is about 30–50 mg per day until blood serum levels reach 60–80 μg/100 cm³ Response of males so injured reproductively is dependent upon the age at which the deficiency became destructive and severity of de generative processes at the time therapy is instigated Response in the female is apparently much quicker and easier to measure

Vitamin E apparently is of value in the single stomach animals however since research workers have shown that this vitamin is synthesized in the rumen (Gullickson *et al* 1944) its use in cattle is a highly controversial sub ject There is considerable clinical data supporting the value of wheat germ

oil wheat embryos sprouted oats and tocopherols given orally or injected and in the everyday treatment of infertility one cannot disregard the many actual case histories where such therapy has brought about improved breeding efficiency

We cannot leave the subject of nutrition and infertility without bringing up the subject of degree of obesity Asdell (1949) proposes that the tendency to fatten readily is indicative of an endocrine imbalance which may also affect fertility Hammond (1952) notes the fact that Samuels reports testosterone as soluble in fat and intimates that this may be a reason for low sex drive and marginal reproductive performance of fat males We must consider the effects of accumulations of fat in the neck of the scrotum covering the epididymis and proximal pole of the testicle acting as an insulation and thus decreasing normal function of the gonads Fatty deposits in the substance of the ovaries and in the periovarian tissue adversely affects the physiological function of the motile bursa during the process of ovulation Mechanical pressure on the delicate uterine tube may be a factor We cannot say that fat sterile cows are sterile because they are fat but we must concede that excessive fatness when the deposits are found to be modifying the anatomy or physiological function of the sex organs is certainly a cause for infertility

Dietary changes must be prescribed and exercise advocated to reduce the total weight in fatness of the animal over a period of time The so-called let down should cover 4–6 months depending upon the reaction of the animal Thyroxin injected or thyroprotein given orally is often indicated

Considering the information available today relative to nutrition and infer tility it is obvious that careful scrutiny of the diet and food element intake should be an important part of every examination of herd infertility problems Even though the cause is later found to be a specific bacterial protozoan or viral infection the status of nutrition of the herd should be assessed and modi fied whenever necessary

Endocrines as related to the treatment of infertility have always been looked at sceptically by research workers because of the difficulty encountered in setting up any type of treatment schedule supported by adequate controls The very nature of endocrines acting as catalysts in a very complex metabolic system confuses the symptomatology However there are sufficient data from both research and clinical workers to support the contention that endo-crines do have a role in the successful treatment of infertility of animals

It is becoming more evident that the sequence of influences of various hor-mones in the female reproductive cycle fall one upon the other in very rapid succession each particular endocrine exerting its maximum influence within a relatively short period One variable in the success of endocrine treatments in the hands of various practitioners is related to timing of the injection relative to the phase of the cycle Hansel and Trimberger (1952) showed that the injection of progesterone 4–5 hours before estrus reduced the average

length of the estrous period almost 4 hours while the same injection, if given more than 2 hours after the beginning of estrus, had no effect upon the ovulation time Uren (1954) states that if PMS is given in the presence of a corpus luteum multiple follicles develop with a tendency toward a cystic condition whereas if the PMS is administered in the absence of luteal tissue the ovary often produces a normal, single follicle Black *et al* (1953) have indicated that forced ovulation during the luteal phase invariably results in low fertility Berliner (1951) has pointed out that estrogens administered in the early part of a heat period can be effective in improving follicular development and ovulation while estrogens given in the last part of the heat period have little or no effect upon ovulation Such information plus practical experience, has led the author to propose this theory relative to treatment with endocrines for teaching purposes or as a thumb rule to assist practitioners in determining when and how much of an endocrine to inject

Let us assume for a moment that the phases of the estrous cycle are the paddles of an old water wheel Each of the twenty one spokes of the wheel represents a day of a normal 21 day cycle The hormone or hormones influential during each phase of the cycle are identified with each spoke Turning at a constant speed then the spokes would pass a given point on the circumference of the wheel at regular intervals of time until a revolution or cycle is completed

The drive shaft brings about the changes in the cellular structure of the reproductive system manifested by follicular maturation the escalator action of the myometrium and uterine tubes ovulation fertilization movement of embryo to the nidatory bed and so on through the necessary stages coincident with and related to the development of a zygote

Now if the revolution were slowed down at any time or it took longer (delayed heats) for the spokes representing any given phase to pass the point then an effect would be brought about in the cellular structures mentioned above Rotation at a faster than normal speed (polyestrus) would also have an effect If this deviation from the normal were severe enough the result might be infertility or non conception or embryonal death And even if the number of days within each cycle were normal perhaps the effect on the drive shaft is not optimum *we could say the clutch is slipping*

The logical treatment would them be to push on a spoke with the proper hormone to regulate the revolving wheel Regardless of which spoke we push if we use the influential hormone for that period of the cycle the whole wheel will be affected For example if we were to give the spoke of proestrum a push by injecting FSH the most influential hormone of that period we would affect all the other phases of the cycle as the wheel revolved If we gave progesterone at the proper time we would affect all the other phases of the cycle as the wheel passed through them It would follow then that the cellular structures influenced by each phase would also be affected the final result being a more

suitable environment for the various phenomena involved in the process of reproduction

This would not agree with the theory advanced that an animal exhibiting regular heat periods could not be deficient in any endocrine (Reece 1954) Such a theory in the author s experience does not hold true Manifestation of clinical heat does not mean that the wheel will follow through each phase with out floundering a single step of the way Therapy should be designed to adjust this balance to that most beneficial to the animal

Casida *et al* (1943) Marden (1952) Black *et al* (1953) have all shown that ovulation can be brought about by administering various dosages and combinations of gonadotrophins Several reports of practical application of this information indicate that gonadotrophins are of value in the practical treatment of infertility (Cameron 1942 Biltz *et al* 1944 Biltz, 1944 Lubin 1946 Hancock 1947 Rasbech 1953 Hill 1954) Luteinizing hormone is used to correct all degrees of polyestrus including nymphomania This hormone usually derived from pregnant women s urine is given at the time of examination of the animal and may or may not be accompanied by manual rupture or vaginal drainage of the cyst Glandular extracts assayed as having an excess luteinizing hormone are effective also Craige (1954) advances the theory that higher blood progesterone levels are required to suppress FSH than to suppress LH Accordingly cystic ovaries may result when the blood level of progesterone is high enough to suppress LH but not FSH thus allowing intermediate follicles to enlarge but ovulation is deterred because of lack of LH 500 mg of Repositol progesterone is thus recom mended as a corrective agent in cystic ovaries manifested by degrees of polyestrus

Critically involved in the complex process of ovulation LH is also given during the latter part of the heat period to force ovulation Because of its con tamination with LTH the hormone influencing the secretion of progesterone habitual abortion in animals may be treated by a series of injections at weekly intervals until the 4th or 5th month of gestation

The FSH usually derived from pregnant mares serum is most effective when used during proestrus or very early estrus (Lubin 1946 Hill 1954) to improve the environment of the developing follicle Apparently its period of influence is limited to that phase of the cycle when luteal tissue has ceased to function but before the heat period has advanced Cows should be examined carefully about the 16th or 17th day of the cycle for absence of luteal tissue before administration of the drug if optimum results are to be obtained It may be used to induce estrus in animals having inactive or infantile ovaries in both the mare and the cow (Cameron 1942)

Low potency estrogens administered early in the heat period to supplement the endogenous estrogen of the developing follicles has been advocated as effective therapy (Berliner 1951)

More recently progesterone has been given attention in the role of infertility and has been injected during the heat period to affect ovulation (Herrick 1953 Dawson 1954 Wiltbank *et al* 1956)

It is evident that the end result of any of the above treatments is to correct an imbalance or to re adjust the quantity of the various hormones involved in follicular genesis and ovulation With reference to the theory of the water wheel advanced above timing of the injection determines the success Use of any one of the four basic hormones mentioned ultimately changes the values of one hormone in relationship to another and therefore modifies the target organ tissues of all the phases of the heat cycle Scrutiny of all the reports of clinical use of hormones indicates that any one of the several will result in practically the same degree of success if given at the proper time in the cycle and in physiological doses Approximately 60–65% of those cases treated will conceive on one or two breedings after treatment

Thyroxine injections in large animals are of course economically impractical except in special cases However the administration of iodides such as Hi amine has been reported as beneficial (Baker 1953) Considering the many physiological benefits of iodides themselves plus the effect on thyroid activity this therapy seems logical (Mason 1948)

Treatment of the anestrous cow is determined by accurate diagnosis since all must agree that the most common cause of anestrous is pregnancy

Confining this discussion to those etiological factors for anestrous which are not infectious we must consider the basic glandular failure as occurring in the pituitary Replacement therapy in the form of FSH (rarely LH or combinations or both) will produce estrus in those animals having ovarian tissue receptive to stimulation by the gonadotrophins In the case of retained active luteal tissue or a static condition of the cycle due to the presence of a corpus luteum active over a long period of time but now non functioning we must resort to stimulation of the pituitary Estrogenic compounds are here indicated

Here again we are applying the theory advanced above that regardless of which spoke we push we are assuming that each other spoke in the wheel will turn also In one instance we are pushing the gonadal wheel by injecting gonadotrophins and in the other case we are pushing the pituitary spoke by injecting steriods The ultimate effect is the same

There is evidence to support the belief that natural estrogens are more likely to produce an ovulatory heat than synthetic estrogen (Rasbech 1953) However from the practical point of view it is advisable to have the animal bred on the heat period following the induced estrus for although a few animals conceive on the artificially produced heat the percentage is not high enough to warrant the added expense of the extra professional call (Gibbons 1951 Easterbrooks 1952)

Enucleation of the corpus luteum as a treatment for infertility in cattle is a

controversial subject It is apparent that the current condition of the corpus luteum as it is embedded in the ovarian stroma at the time of examination should dictate the procedure used Jakobsen (1956) reports no adverse effect of 2746 enucleations of the corpus luteum periodicum removed on the tenth to twelfth day following a normal heat period Such a corpus luteum can be removed quite readily in its entirety by exerting a slight pressure on the luteal tissue (not on the ovarian stroma) along the line of demarkation However those corpora lutea which have been present for considerable periods of time (post parturient retained C L) often are covered by a thick fibrous capsule and are palpated as a smooth elevation protruding above the normal contour of the ovary Other corpora lutea are large and irregular and give the impres sion of being fibrous in nature Attempted enucleation of these fibrous deeply imbedded corpora lutea often results in shredding or splitting remnants re maining in the cavity Such structures should not be enucleated manually under any conditions Hemorrhage and fibrous adhesions of bursa and peri ovarian tissue blood leaking into the uterine tubes and eventual immobilization of the fimbria and ovary and possibly portions of the uterine tube may result (Hill 1956 Moberg, 1956) It is not contested that enucleation of the corpus luteum is an effective therapy The incidence of success in treating anestrus is as high as any other known technique and the percentage of conceptions fol lowing enucleation of the corpus luteum perodicum is as high as with any therapy known Judicial practice of enucleation of the corpus luteum may be condoned however the effectiveness of long acting estrogens such as ECP and Repositol type estrogens precludes the necessity for expressing every corpus luteum which is incriminated in anestrus or repeat breeder cows

Uterine distention with warm sterile PSS is reported as a treatment for anestrus in the mare although it is of little value in other animals (Roberts 1956)

Hays and Carlevaro (1956) report a respectable degree of success in treat ing anestrus cows with electrical stimulation the estrus occurring 1–7 days after stimulation and a corpus luteum forming in each case This therapy should be investigated more extensively as the theory has been applied for centuries in the form of massage per rectum and is still advocated by many veteran sterility experts

The incidence of embryonal death is alarmingly high in cattle (Tanabe and Casida 1949 Casida 1953 Tanabe and Almquist 1953, Hawk *et al* 1955) This phenomenon which usually is manifested by one or more normal pre service cycles and then return to estrus at prolonged intervals of time after service to a known fertile sire is the most annoying abnormality of animal reproduction Apparently it occurs in all mammals including humans The causes advanced are many and of course, physiological function from matu ration of the ova in the female maturation of the sperm in the male union of the two gametes to produce the zygote cleavage and development of the zygote

as related to its inherent activity plus the environmental effects which the young embryo is subjected to during its early life prior to permanent attachment of the placenta present many stages where the embryo is vulnerable and may succumb Treatments prescribed include bacteriocidal agents endocrine dietary and systems of mating

In the light of present day knowledge treatments for embryonal death would probably be most effective in this order First, infusion of the uterus with antibiotics secondly, endocrine replacement therapy in the form of luteinizing hormone administered during late estrus during metestrus and continued into diestrus to strengthen the corpus luteum and stimulate its function to a higher degree until placental function is adequate to hold the pregnancy Progesterone given at weekly intervals after conception has occurred is also of value (Woelffer 1951 Herrick 1953 Craige 1954) Thirdly correction of adverse dietary factors and fourth changing the sire used

From the wealth of information obtained, probably the greatest single cause of death of the embryo in animals is a low grade infection of the uterus commonly called endometritis This pathological condition is difficult to diagnose positively since there are no abnormal discharges noticeable Sometimes there is a slight abnormality in tone and resilience of the uterus during estrus which can be detected rectally Bacterial culture of the endometrium may reveal any one of several of the common pathogenic organisms The ovarian cycle is not modified

Intrauterine injections of antibiotics have been reported by many workers (Woelffer 1951 Chambers 1952 Fincher 1952 Lindley 1954 Sacchi *et al* 1957 Roberts 1956 Hienze 1957) with a variation in time of infusion relative to time of service Injection just prior to breeding 15–30 minutes after a service 6–12 hours post service or infusion on one heat period with service on the following heat are all of value Recently Sacchi *et al* (1957) reported enviable success with low level antibiotic isotonic douches of calcium–oxytetracycline*–glucose complex injected through the same instrument used to inseminate the cow immediately after the semen was expelled

Hienze (1957) reports that routine intrauterine treatment of parturient cows with antibiotic boluses did not improve breeding efficiency but in fact the animals so treated required more services per conception than untreated controls

The volume of sterile water or physiological salt solution or sulfonamide solution used as a vehicle apparently is not critical but 10–25 cm³ is sufficient Evidence is available to point up the fact that semen from certain bulls particularly low breeding efficiency bulls when used on cows for first service may adversely effect conception when semen from different bulls is used on repeat services (Christian *et al* 1951 Flerchinger and Erb 1953 Hawk *et al* 1955) Veterinarians inseminators and animal husbandmen have often reported that

* Trade mark of Chas Pfizer and Co Inc Terramycin

mating a shy breeding cow to a bull of another breed resulted in conception however careful study of the phenomenon (Christian *et al* 1951 Corley *et al* 1952) reveals that it is not cross breeding *per se* which is beneficial but that there is usually a difference in bull fertility or the successful conception is due to elimination of this incompatibility factor mentioned above

The effectiveness of treatment of infertility in animals and humans alike is severely inhibited because of our inadequate methods of diagnosis Our in adequate methods of diagnosis often hinge upon our meager knowledge of the over all complex phenomena of reproductive physiology in either the male or female It seems evident now from the great store of information available that there is no single panacea for the problem of infertility but that successful therapy must include all the facets of influence Our therapeutic instruments can advance only as rapidly as our knowledge of the subject Investigations must continue on the influence of pituitary–gonadal relationship we must unveil the secrets of the other glands such as the adrenal the thyroid and per haps the uterus itself as a gland and their influence on reproduction Combi nations of hormones mixture of drugs and/or dietary factors as therapeutic measures should receive careful consideration

Although we do have available a limited armamentarium the effectiveness of which is dependent upon experience in diagnosis our greatest need is to find practical workable methods of detecting the site and/or cause of the pathology which can be applied in the everyday sterility practice

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CLASSIFICATION OF MALE HYPOGONADISM

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THE first attempts to classify the various forms of male hypogonadism were made in U S A Heller and Nelson (1948) and Howard *et al* (1950) used the gonadotrophin excretion in the urine as base for their classification while Albert *et al* (1953) believed that morphological criteria would be the best way for exact differentiation and classification of testicular disorders However we still do not yet possess a reliable and all-embracing classification of every type of male hypogonadism The reason for this is that a variety of potential factors are responsible for the final clinical picture The most important of the e are

1 The fact that the male gonad has two functions—an excretory and incretory one—which can be damaged separately and independently
2 The intensity of the damage can vary from an only slight to a very considerable one
3 The disorder or lesion may start in pre or post puberal time
4 The site of the defect may be located in the testicle itself or in other organs e g in the pituitary the hypothalamus the adrenal cortex and elsewhere

To classify the various forms of testicular disorders the clinician uses may diverse clinical and laboratory criteria Of the latter may be mentioned only the microscopical and chemical examination of the ejaculate the testicular biopsy determination of gonadotrophins and steroidhormone metabolites in the urine In discussing the classification it seems important to recognize the value of the various laboratory methods but also first of all to review the criteria of normal testicular function

At the first clinical examination it is important to note the size and consistency of the testicles although a normal finding does not indicate that they are functioning adequately This point needs emphasis because it happens sometimes that for example the success of an orchidopexie has been considered only with regard to a normal position and the postoperative size and consistency of the gonads This conclusion is erroneous however since an exact analysis of the ejaculate of operatively treated cryptorchids may show

severe defects in spermatogenesis (Hansen 1946 1949) For a long time the microscopical examination of the ejaculate has played the most decisive role in the diagnosis of normal or defective testicular function Indeed a change in the sperm number and quality gives good information concerning the functional state of the tubules although such finding naturally does not exclude the possibility of pathological changes in efferent seminal ducts A normal count is assumed when over 60 mill sperms per ml with 70–85% normal forms are found The percentage of mobile sperms varies from 50–90% the average being approximately 70% Based on a critical analysis of 536 ejaculates of normal males Simmons (1947) has shown however that even in cases with sperm counts over 100 mill about 30% may be found with pathological forms and insufficient motility Below 60 mill the quality of the ejaculate progressively declines In spite of all the criticism one can raise against normal values of sperm counts in the human one cannot deny the fact that the spermiogram reflects precisely the functional condition of the tubules From this we conclude that the microscopical analysis of the ejaculate is really the most reliable criterion of tubular function Cases with pathological changes in efferent siminal ducts resulting in lowering of the sperm number can be excluded by testicular biopsy

The next question is how to obtain objective criteria about the Leydig cell function *i e about the production of testosterone presents a much more* difficult problem Normal development of sex characters and normal size consistency and secretion of the accessory sex glands are undoubtedly very useful criteria It must be kept in mind however that the secondary sex characteristics once developed become relatively independent of the circulating testicular hormone as shown by the experience in late castrates A somewhat more sensitive index of testosterone production is the status and function of the accessory sex glands and these organs are therefore from the diagnostic standpoint more reliable indicators than the secondary sex characters

But here also size and consistency of prostate and/or the amount of seminal fluid give only indirect approximative data Biological assays and chemical techniques have therefore been introduced to measure the presence and amount of testicular androgen With these endeavours we encounter the difficulty of separating the androgens produced in the testis from those deriving from *the adrenal cortex Such separation is practically impossible* (Voigt and Nowakowski 1957) In the last few years the interest has been focused on the chemical assay of the seminal plasma Mann (1955) observed that the sugar content in the ejaculate is not glucose but fructose produced by the epithelium of the seminal vesicles and maintained that secretion of this fructose is closely related to the Leydig cell function *On the basis of this relationship he de*veloped a new quantitative test for the estimation of testosterone production It is however still an open question whether the results of Mann s animal experiments can be applied to human beings If it could be proved the

clinician would have a simple and sensitive method of getting the information which he needs about testes endocrine function

The normal values of the fructose concentration in healthy and sexually normal developed males published by different authors are shown in Table I

TABLE I

Fructose Concentration in Human Seminal Plasma

(Males with normal sperm counts)

	Number of tests	Fructose γ/ml
Harvey (1948)	123	50–6400
Eichenberger and Goossens (1954)	5	700–3700
Landau and Loughead (1951)	24	2100–8070
Raboch and Hradec (1954)	24	1390–5290
Gropper and Nikolowski (1954)	19	1500–4000

There is relatively good agreement between Raboch and Hradec (1954) and Gropper and Nikolowski (1954) in regard to their normal concentrations The minimal values found by Harvey (1948) and Eichenberger and Goossens (1954) are much lower

To divide whether these extremely wide variations are physiological I have in collaboration with Schmidt (in press) studied the fructose concentration with respect to age

In Fig 1 are entered the fructose-concentrations of 83 normal males be

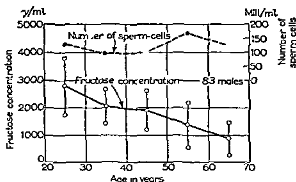


FIG 1 Fructose-concentration of the ejaculate in 83 healthy normospermic males

tween the ages of 20 and 70 years For each decade the average values and standard deviations were calculated The corresponding average values of the sperm counts are also shown graphically It is obvious that the fructose concentration falls with progressive age in a statistically significant manner Between 20 and 30 years the average fructose level is about 2800 γ/ml and only 800 γ in the sixth decade of life which is one third of the younger group The decline of the fructose concentration with progressing age has the same correlation as the excretion of androgens in the urine determined biologically or chemically If one considers further the standard deviations in the youngest and oldest group it becomes quite evident why fructose determinations in the human seminal plasma yield such wide normal variations (between 300 and 3800 γ/ml) Thus these variations are no argument against the use of this method as an index of androgenic activity in man since they can be explained by diminished testosterone production in the Leydig cells with progressing age We will return to this point later but may conclude here that a close relationship between the fructose content in the ejaculate and testicular androgen production exists and that fructose determinations are a very useful clinical index of Leydig cell function In this connexion it is of interest to know that the relative number of the Leydig cells and their lipid content decreases progressively with age—as recently shown by Tillinger (1957) and Lynch and Scott (1942)

Male gonadal insufficiency or hypogonadism can be defined as insufficiency of testicular function in regard to sperm and testosterone production But since the testis has a double function it is essential to differentiate clearly between tubular—and hormonal insufficiency

The diagnosis of tubular insufficiency is easy because a damage of the tubules affects spermatogenesis and is associated with a fall of the sperm quantity and quality Oligospermia may be considered the earliest symptom of tubular insufficiency It is accompanied by characteristic changes in the microscopic structure of the testis If the disease progresses the sperm number falls continuously until azoospermia or aspermia are reached These different stages of tubular insufficiency are characterized by typical pathological findings in specimen taken by testicular biopsy

If oligospermia is not very severe the tubules are of normal size The epithelium looks quite normal and there is only sloughing of immature sperm cells into the lumina (FIG 2a) Figure 2b is obtained from a patient with severe oligospermia The tubules show more degenerative changes with slight peri tubular fibrosis and disorganization of the epithelium Regionally completely atrophic tubules may be seen

Further progress of the disease results in azoospermia and in this stage the tubules contain only Sertoli cells In some parts remnants of sperm cells can be detected The peritubular fibrosis is much more severe and sometimes proliferation of Leydig cells may be seen

(a)

(b)

FIG – Testicular structure in mild (*a*) and severe (*b*) oligospermia (160 hematoxylin-eosin)

Much more difficult than the diagnosis of tubular insufficiency is the detection of hormonal insufficiency of the gonad If typical eunuchoidal (early or late) symptoms govern the picture diagnosis is easy But difficulties are greater if regression of secondary sex characters is lacking and only certain subjective complaints point to deficient testosterone production e g. in the so-called male climacteric In such cases fructose determinations are useful to get objective information about the functional state of the Leydig cells

The experiments and conclusions of Mann and his group that the fructose concentration is a reliable index of testosterone production in the animal cannot be doubted and have been confirmed by Gassner (1952) but its validity had to be demonstrated for human beings by showing (1) that in sufficient testosterone secretion is accompanied by fall of fructose-concentra tion and (2) that the latter can be raised by testosterone administration We examined this problem with Schirren (Nowakowski and Schirren 1956) in 31 aspermic patients who were divided into two groups in the first aspermia was the result of bilateral occlusion of seminal ducts in the second group aspermia was of testicular origin In the later group two subgroups could be separated one with normal size of the prostate the other with marked prostatic atrophy

TABLE II

Aspermia with Bilateral Occlusion of Seminal Ducts

n—normal r—right l—left

			Testes			Prostate	Ejaculate			Fructolysis
No	Name	Age	r	l	Histology	Size	Fluid ml	pH	Fructose γ/ml	
1	H Fi	29	n	n	—	n	2.5	7 8	2300	—
2	W Kr	30	n	n	n	n	2 5	7 7	2576	—
3	A Sch.	31	n	n	—	n	2 5	7 6	2100	—
4	K. Gu	33	n	—	—	n	3 0	7 4	1776	—
5	F Ma	37	n	n	—	n	2.5	7 8	4640	—
6	J Si	38	n	n	—	n	4 5	7 8	2300	—
7	H Si	40	n	n	—	n	3.5	7 8	1800	—
8	C Ze	40	n	n	—	n	4 0	7 4	1344	—
9	B Bl.	44	n	n	n	n	3 0	7 8	1200	—
10	H Gr	46	n	n	—	n	1 0	7 6	3680	—

From Table II it will be seen that in cases of aspermia due to bilateral occlusion of seminal ducts but normal functioning testis the fructose content is normal and in good correlation with normal size and consistency of the prostate

TABLE III

Aspermia with Bilateral Primary Testicular Atrophy

(Size of prostate normal)

			Testes			Prostate	Ejaculate			FSH	17 Kst
No	Name	Age	r	l	Histology	Size	Fluid ml	pH	Fructose γ/ml	MU/day	mg/day
11	K K	42	+++	+++	3	n	1 0	7 2	2760	96	16 40
12	W Go	31	+++	+++	2	n	1 2	7 4	2500	26 4	21 30
13	K Oe	45	++	++	—	n	2 5	7 6	1300	—	—
14	O Bo	31	++	++	2	n	1 0	7 8	1380	96	11 41
15	K We	36	++	++	2	n	1 2	8 0	3600	26 4	3 16
16	K S h	24	++	++	2	n	4 5	7 2	3760	192	8 9
17	G Ha	22	+	+	2	n	1 0	7 4	2576	—	—
18	A Ho	32	+	+	2	n	3 0	7 8	3700	—	—
19	H Ga	31	+	+	—	n	3 0	7.4	1480	192	—

Key + + + slight
+ + moderate } testicular atrophy (macroscopically)
+ marked
r = right
l = left
1–4 Grade of atrophy microscopically Classification according to Staemmler (1942)
n = normal FSH = urinary follicle stimulating hormone
17 Kst = urinary 17 ketosteroids MU = mouse units

Table III summarizes our findings in patients with aspermia of testicular origin Here we found again normal fructose levels the secondary sex characteristics in this group were normally developed, and size and consistency of the prostate was also normal in spite of the atrophic testis In the next group of patients with far more severe testicular atrophy (sometimes we found only connective tissue) a significant lowering of the fructose content was recorded (Table IV) As a matter of fact sometimes no trace of fructose could be detected Some of these patients had typical eunuchoid symptoms and in all cases the prostate was markedly atrophied To 8 of these patients testosterone was given resulting in a significant rise of the fructose content to normal levels (see FIG 3 Pat C Fe Nr 22)

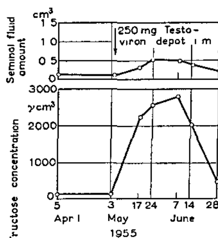


FIG 3 The effect of one single i m injection of 250 mg testosterone oenanthate on the fructose-concentration and the amount of seminal fluid in a case of aspermia with primary testicular atrophy (Pat C Fe see Table IV case No 22)

Table IV

Aspermia with Bilateral Primary Testicular Atrophy

(Prostate atrophic)

			Test			Pro t t	Ejac late			FSH	17 K t
No	Name	Ag		1	Hist l gy	S ze	ml	pH	F close γ ml	MU/day	mg/d y
0	H Ba	44	++	++	3	++	3 0	7 8	0	96	—
21	O Sch	48	++	++	2	++	3 2	7 6	0	96	10 09
2	C. F	22	+	+	3	+	*0 2*	*7 4*	*0*	*488*	*14 75*
23	W W	35	—	+	—	+	0 5	—	0	88	15 43
24	L R	25	+	+	4	+	0 3	7 4	512	192	10 97
5	R Sch	32	+	+	4	(+)	1 5	7 2	3 0	384	5 3
6	K Ha	37	+	+	4	+	1 1	7 4	7 0	192	9 39
27	M A	54	+	+	4	+	1 2	7 8	608	96	8 3
28	A La.	30	(+)	—	—	++	2 0	7 6	950	384	14 20
29	H Pr	33	+	+	—	++	2 0	7 9	360	96	13 78
30	P J	57	+	+	—	++	0 6	7 6	46	96	15 82
31	Ze	34	+	—	—	++	1 0	7 4	40	—	—

Key + + + slight
+ + moderate } testicular resp prostatic atrophy (macroscopically)
+ marked
r = right
l = left
1–4 Grade of testicular atrophy microscopically (Classification according to Staemmler (1942))
FSH = urinary follicle stimulating hormone
MU = mouse units
17 Kst = urinary 17 ketosteroids

From these results it must be concluded that in patients with aspermia of testicular origin the fructose content of the seminal plasma is a reliable index of testosterone production and that a significantly low concentration indicates insufficient hormonal function

In the same way we studied the effects of testosterone on the fructose level in normospermic males If the assumption we made was right that the fall of the fructose content with progressive age is the result of diminished testo sterone production we expected a rise of fructose level in the elder groups approaching the normal range of younger individuals Figure 4 demonstrates the effect of testosterone phenyl propionate in a 65 year old man the fructose rises to normal levels before the reduction of the sperm number begins After discontinuation of hormone administration the fructose level remains normal for a long time and is independent of the sperm number which rises in a typical rebound manner

We treated 17 older patients in this way 13 of them (=76%) showed the expected positive reaction One of the remaining four cases received an in sufficient dosage of the male hormone In the other three cases we are presently unable to decide if the negative result is due to one or more other contributary factors

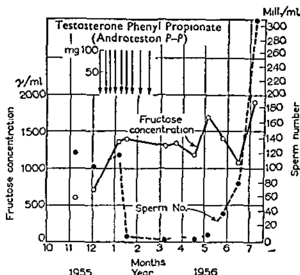


FIG 4 The effect of testosterone-phenylpropionate on the fructose-concentration and sperm number in a 65 year old man After a rise of the fructose to normal levels the depression of the sperms begins After discontinuation of the hormone treatment typical rebound effect

Summarizing our results we conclude that exact differentiation and classification of tubular and hormonal insufficiency of the testis is possible by the microscopical and chemical analysis of the ejaculate

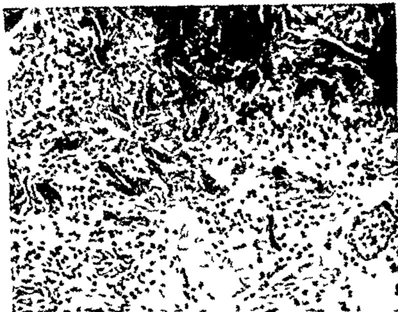
Referring to the disturbed tubular or hormonal function of the testis we can classify the various forms of male hypogonadism in different groups as seen in Table V where we have furthermore listed the different stages of tubular

TABLE V
Classification of Testicular Disorders
(after Hellinga)

Leydig cell function	Tubular function		
	Normal	Deficient	Absent
Normal	Normal male	Oligospermia *(of testicular origin)*	Azoo resp aspermia
Deficient	Male climacteric	Tubular + hormonal deficiencies	
Absent	Fertile eunuchs		(*a*) Castration fibrosis testis (*b*) Idiopathic eunuchoidism



(a)



(b)

5 Different stages of primary testicular atrophy (a) beginning (b) complete atro[illegible] proliferation of Leydig cells is to be seen (a ×160 b ×77 azan)

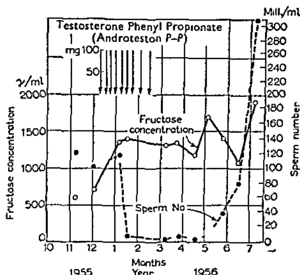


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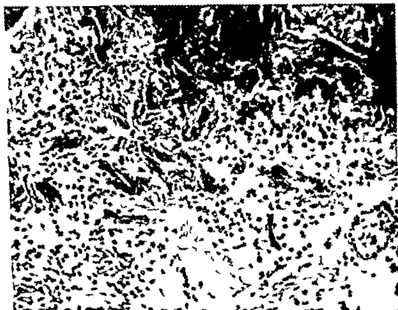
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Deficient	Male climacteric	Tubular + hormonal deficiencies	
Absent	Fertile eunuchs		(a) Castration fibrosis testis (b) Idiopathic eunuchoidism



(*a*)



(*b*)

FIG 5 Different stages of primary testicular atrophy (*a*) beginning (*b*) complete atrophy In FIG 5*a* proliferation of Leydig cells is to be seen (*a* ×160 *b* ×77 azan)

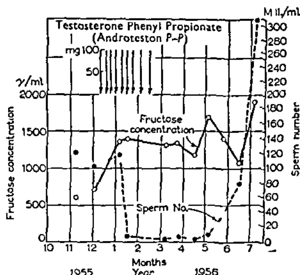


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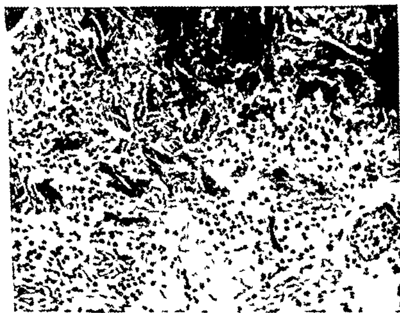
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Deficient	Male climacteric	Tubular + hormonal deficiencies	
Absent	Fertile eunuchs		(a) Castration fibrosis testis (b) Idiopathic eunuchoidism



(a)



(b)

*ferent stages of primary testicular atrophy (a) beginning (b) complete atrophy
...feration of Leydig cells is to be seen (a ×160 b ×77 azan)

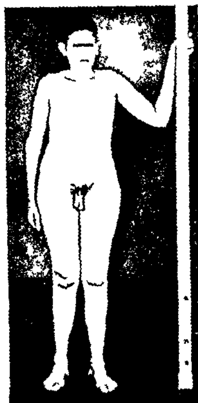
(a)

(b)

FIG 6 Different stages of secondary testicular atrophy (a) beginning, (b) complete atrophy Note no well-developed Leydig cells to be detected (160 azan)



(*a*)



(*b*)

Fig. 7 Idiopathic selective pituitary failure with deficient gonadotrophin secretion in the male

(*a*) Idiopathic eunuchoidism (Pat K. H. [illegible]5 years)

(*b*) ICSH deficiency" fertile eunuch (Pat H. Schu[illegible] 25 years)

FIG. 8 Histology

and hormonal insufficiency from normal to deficient to absent In this arrangement it may be seen that

1 In the adult healthy male the production of sperm cells and testosterone is normal
2 The most extreme stages of tubular and hormonal deficiency are encountered in
   (*a*) castration and complete fibrosis of the testes and
   (*b*) in idiopathic eunuchoidism and complete destruction of anterior pituitary in which FSH and ICSH production is completely lacking

Between these two extremes we can find all variations

3 Such as the male climacteric as an example of selective Leydig cell deficiency and
4 The fertile eunuchs which represent the extreme stage of the latter

The result of isolated damage of tubular function is

5 *Oligospermia* or
6 Azoo resp aspermia of testicular origin

In the remaining squares we can fit in all other cases with disturb d tubular and hormonal deficiencies Such cases we will find especially in patients with selective pituitary failure and deficient gonadotrophin secretion but also in cases with primary hypogonadism where tubules and Leydig cells are involved

A further step towards a satisfactory classification is the age of the patients

(*a*) If the testicular dysfunction begins postpuberally that means after complete development of secondary sex characters the clinical signs of hormonal deficiency are not very severe and sometimes only subjective complaints point in this direction If the hormonal deficiency is of longer duration the clinical picture of late eunuchoidism develops
(*b*) Prepuberal beginning leads to the classical picture of early eunuchoidism The clinical signs are so well known that I need not discuss this in detail
(*c*) The destruction of the male gonad in the fetal period leads to different clinical syndromes One typical example is the Turner s syndrome provided the chromosomal sex in these cases is of the male type

In general we may differentiate between primary and secondary hypogonadism In instances of defects within the testis the condition is regarded as primary hypogonadism When the defect can be shown to be failure of the production or release of gonadotrophic hormones or a dissociation of hypothalamic control over the testis the condition is considered to be secondary hypogonadism

'o distinguish between these two forms, two methods are at our disposal estimation of gonadotrophin excretion in the urine and the testicular psy 17 Ketosteroid determination and fractionated steroid hormone lysis are necessary to get information about the adrenal function

n spite of the criticism which may be and has been levelled at the different hods recommended for the assay of gonadotrophins the estimation of ad stimulation hormones has been widely used by clinicians for the racterization of primary and secondary hypogonadism

Ieller and Nelson (1948) classify male hypogonadism in hyper and hypo adotrophic forms The hypergonodotrophic syndromes are of primary and hypogonadotrophic cases of secondary origin Yet it is well known that ietimes one meets cases with extreme tubular and hormonal insufficiency, which gonadotrophin excretion is normal Therefore Howard *et al* (1950) irated three groups of testicular insufficiency with low normal or high adotrophin excretion It seems to be important that the second group tains very different clinical material intra and supra sellar pituitary ors oligospermia of testicular origin and oligospermia subsequent to oc ion of seminal ducts From this it must be concluded that gonadotrophin etion alone cannot be considered a sufficient clue for differential diagnosis

esticular biopsy is also a very useful method Before I consider its role for differentiation of primary and secondary hypogonadism some preliminary arks seem necessary Recently Getzoff (1955) sent questionnaires to ous members of the American Society for the Study of Sterility and the logical Society and received surprisingly negative answers about this hod But early in 1947 Charny (1947) one of the pioneers and experts in field presented a critical five years survey He stated that in cases with 'ertility or sterility of testicular origin there is good agreement between m number and biopsy findings If this is not so a lesion of the seminal way must be assumed But the value of testicular biopsy is not restricted xplain cases with subfertility and sterility of testicular origin it is also ible to differentiate with the help of the histological findings primary and ndary hypogonadism These two groups can be separated by the con n of the Leydig cells In cases of advanced primary testicular atrophy the ig cells may have more or less proliferated whereas in secondary hypo-idism the atrophy of the Leydig cells is the most characteristic histological ng A subgroup of secondary hypogonadism is represented by the im ire testicle (e g in idiopathic eunuchoidism) owing to insufficient hypo eal stimulation during puberty

iese statements show that the use of histological criteria enables the cian to get more precise information with regard to differentiation een primary and secondary hypogonadism especially in patients where gonadotrophin secretion is normal Apart from this it is possible in this to get prognostic information in regard to hormone treatment

In FIGS 5a and b different stages of primary testicular atrophy are illustrated The earliest stages were demonstrated just above in the discussion on oligospermia (see FIG 2) The section in FIG 5a has been taken from a case of advanced peritubular fibrosis The tubules contain only Sertoli cells In the intertubular tissue excessive proliferation of Leydig cells occurs

If the atrophy progresses further the tubules obliterate completely and in this stage degenerative changes in the proliferated Leydig cells occur The final stage is complete fibrosis of the testes Very different are the pictures in secondary testicular atrophy Figures 6a and b demonstrate two different stages In the first case a necrosis of the pituitary was present in the other an intra sellar chromophobe adenoma has been found In none of these cases have reasonably developed Leydig cells been detected

The analysis of the steroid hormone pattern in the urine is a further step in the differential diagnosis especially in those cases in which the adrenal cortex is involved (see FIG 10) Summarizing it can be said that exact differentiation between primary and secondary hypogonadism is only possible with the help of all the methods mentioned But it should be pointed out that sometimes cases present themselves in which it is impossible to classify them precisely in one or another group in spite of the use of all tests

Some clinical aspects of male gonadal insufficiency need discussion to prove the value of the different diagnostic procedures for the classification of male hypogonadism With this aim in mind it seems very useful to give a survey of our clinical material observed in the last six years at the 2 Medical Clinic of the University in Hamburg because this gives a good idea of the frequency of the different forms of male hypogonadism

Our material comprises 132 cases with aspermia of testicular origin Two thirds are of primary one third only of secondary origin The proportion changes in favor of the former if patients with oligospermia and selective Leydig cell failure of testicular origin are added to the first group

TABLE VI

Primary Hypogonadism

	Number of Cases
Surgical castration	4
Functional prepuberal castration	10
Cryptorchidism (bilateral)	36
Klinefelter s syndrome	3
Bilateral testicular atrophy	
Traumatic	1
Infectious	1
Dystrophia myotonica	8
Unknown etiology	22
	85

In primary hypogonadism (Table VI) testicular atrophy resulting from cryptorchidism occupies the first place This is followed by cases with bilateral atrophy of unknown etiology and functional prepuberal castrates It demonstrates clearly the high importance of cryptorchidism as a cause of male sterility Furthermore it can be concluded from this survey that in the majority of the other cases with primary testicular atrophy nothing can be said with regard to etiology This is also true for oligospermia of testicular origin, in which we see the first stages of primary atrophy In dystrophia myotonica the observed testicular atrophy belongs to primary hypogonadism because we found, together with Mertens and Nowakowski (1954) the same histological changes as in other cases of primary atrophy

In the group with secondary hypogonadism (Table VII) patients with idiopathic selective pituitary failure and deficient gonadotrophin secretion are the most prevalent They represent in our material over 50% of all patients with secondary hypogonadism

Table VII

Secondary Hypogonadism

	Number of Cases
Idiopathic selective pituitary failure with deficient gonadotrophin secretion	27
Adrenogenital syndrome	4
Hemochromatosis	2
Pituitary tumors	8
Pituitary necrosis	2
Hypothalamic tumors	3
Transection of spinal cord	1
	47

With the aid of gonadotrophin assay and testicular biopsy different subgroups of partial gonadotrophic insufficiency may be separated as shown in the survey presented on Table VIII The first group is made up by those patients who concluding from hormone analysis and biopsy findings produced neither FSH nor ICSH To this group also belong the so called idiopathic eunuchoidism the adrenogenital syndromes and intrasellar pituitary tumors with complete lack of both gonadotrophins Apart from this group we have to separate patients with an isolated lack of ICSH the so called fertile eunuchs (McCullagh *et al* 1953) In the first group no detectable amounts of gonad stimulating hormones are to be traced in the group with ICSH deficiency normal or subnormal titers are to be found With the help of gonadotrophin assay (and testicular biopsy) it is not only possible to differentiate between primary and secondary hypogonadism but also to separate the latter group into one with FSH and ICSH deficiency and into another with lack of ICSH only



FIG 9 Isosexual adrenogenital syndrome with congenital adrenal hyperplasia in the adult male

(*a*) Pat G K 25 years Clinically stunted growth aspermia dissociated virilism



(*b*) Testicular biopsy of the same patient under developed tubules with spermatogenic arrest slight fibrosis of the tubular walls No Leydig cells in the inter tubular tissue

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TABLE VIII

Selective Pituitary Failure in the Male

Deficient Gonadotrophin Secretion

FSH+ICSH	ICSH
A	
Prepuberal boy Idiopathic eunuchoidism Adrenogenital syndrome Pituitary tumor (intrasellar craniopharyngeoma)	Fertile eunuchs
B	
Oestrogentherapy	Secondary idiopathic Leydig cell failure in the adult
Hemochromatosis Pituitary tumor	Pituitary tumor (chromophobe adenoma)
No detectable gonadotrophins in urine (<6 6 MU/day)	*Normal resp subnormal gonadotrophin-levels in urine*

A Prepuberal B Postpuberal

From these prepuberal forms (A) we divide the postpuberal cases (B) as shown in Table VIII

It now remains to discuss the diagnostic problems in unitrophic hypophyseal deficiency and this will be done by illustration of some selected cases

Figures 7a and b show two patients of the same age with marked sexual infantilism One man (*a*) with idiopathic eunuchoidism shows hypogenitalism + hypogonadism because the size of the testicles was not bigger than a pea But in the other (*b*) also with extreme hypogenitalism the testes had approximately normal size and consistency The most important fact here is the dissociation between the size of the penis and that of the testes that means the hypogenitalism without remarkable hypogonadism

In FIGS 8a and b the histological findings of testicular biopsy specimen are presented An extreme underdevelopment of the tubules and Leydig cells is evident in the case with idiopathic eunuchoidism (*a*) but approximately normal size and differentiation of the tubules and their epithelium are found in the other patient (*b*) In the first patient the hormone analysis yielded no gonadotrophins in the urine whilst in the other the laboratory reported normal amounts

This example demonstrates that it is possible to differentiate the two subgroups of partial gonadotrophic insufficiency by application of a microscopic study and hormone analysis and combining the results with the clinical examination

The next example is a case of male gonadal insufficiency resulting from bilateral adrenal hyperplasia which I first described in 1952 with Puschel (Nowakowski and Puschel 1952) This 25 year old man (FIG 9a) requested medical advice because of stunted growth and sterility Besides the stunted growth the study of this patient revealed aspermia and a dissociated development of the sex apparatus The size of the penis was normal and no lack of secondary sex characteristics could be diagnosed but the testes were extremely small and the biopsy specimen manifested the typical picture of infantile tubules and immature Leydig cells as well as some degree of tubular fibrosis (FIG 9b)

The hormone analysis uncovered a high excretion of 17 ketosteroids but a lack of gonad stimulating hormones in the urine While under treatment with cortisone the high 17 keto excretion was reduced gonad stimulating hormone appeared in the urine and growth and development of the testes was observed (FIG 10)

This case seems of interest because it demonstrates that classification of hypogonadism by aid of gonadotrophin assay alone is insufficient With the slightest suspicion of hyperfunctioning adrenal cortex determinations of 17 ketosteroid becomes mandatory

Studying with Schirren (Nowakowski and Schirren 1956) and Schmidt (Nowakowski and Schmidt in press) the relationship of sperm plasma-fructose and Leydig cell function we sometimes observed in normospermic males (as mentioned above) extremely low fructose-concentrations in the ejaculate of the same grade as in cases of aspermia with bilateral testicular atrophy and hormonal deficiency If these patients reacted favorably on testosterone application the diagnosis of selective Leydig cell failure was made In some cases it was possible to treat them—after discontinuation of testosterone and fall of the fructose to the pretreatment level—with HCG

It can be demonstrated from FIG 11 a rise of the fructose content after HCG application was obtained Schirren (personal communication) observed more cases of this kind who all reacted favorably to HCG treatment From this I conclude that in cases with postpuberal Leydig cell deficiency there are some of secondary origin To differentiate them from primary Leydig cell insufficiency seems only possible with the help of HCG

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G K ♂ 25 J

Cort sone acetate mg/d y
Ees oph ls ml

Gonadotrophins MU/24 hr

Cortin ds mg/24 hr

17 Keto-ster ds mg/24 hr

Feb March Apr l May June July August Sept
1953

Fig 10 The effect of cortisone on the 17 Kst and gonadotrophin excretion in a case with isosexual adrenogenital syndrome with congenital adrenal hyperplasia (see Fig. 9)

1 Reduction of 17 Kst-excretion
2 Appearance of FSH in urine and
3 Growth of testes—all under cortisone treatment

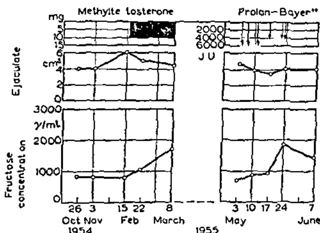


Fig 11 The effects of methyltesto terone (left) and HCG (right) (Prolan Bayer) on the fructose concentration in the seminal plasma in one patient with secondary postpuberal Leydig cell insufficiency the fructose-content of the ejaculate rises after testosterone and after HCG

DISCUSSION

Wednesday July 3 1957

Morning Session

E P REINEKE, Presiding

GENERAL PROBLEMS OF REPRODUCTION

W E. PETERSEN It is perfectly obvious from Dr Donker s presentation that the problem of regulating estrus in cattle is quite complicated and is far from solved There are a number of important phases that need to be investigated by competent workers In going through the literature published since the II Symposium was held two years ago we were rather dismayed by the fact that new evidence in this direction is scarce mainly because the number of people working in this field is quite small in spite of the fact that for instance application of such information in artificial insemination could have a real practical value With regard to the complexity of this problem may I call your attention to the fact that we in Minnesota have diligently tried to transplant fertilized ova and have completely failed to accomplish this One of the reasons for this as Dr Donker pointed out is our inability to synchronize the responses of donor to recipients It also appears that much fundamental work must be done to point up the reasons for the apparent low fertility during estrus following the withdrawal of progesterone Since we are unable on the basis of the knowledge presently available to determine the reason for this I certainly would strongly support Dr Donker s suggestion that rather competent and detailed histochemical studies of the entire reproductive tract should be done to ascertain just why fertility is reduced in the heat following progesterone treatment. I should like to call your attention also to the fact that we at Minnesota have initiated a rather substantial project in which progesterone is administered to all cows We then attempt to ascertain on a large scale the status of fertility following services at the first estrus and subsequent ones It is unfortunate however that at the present time we do not have the facilities nor the man power to permit us to carry on detailed histochemical studies of the various parts of the reproductive tract that may be affected

H J HILL Will the heat periods following the first estrus occurring after progesterone treatment fall within a few days of the normal cycle or do they become scattered again?

J D DONKER It appears that each animal has a more or less characteristic estrous pattern While we concede that the normal duration of the estrous cycle to be between 17 and 24 days it is quite likely that if a cow cycles normally at 17 days that it would cycle again at 17 days On the other hand many cows will establish a new pattern after treatment and depart from that previously shown In other words at the second estrus following withdrawal of progesterone one can expect that the estrous period is spread over a much wider time.

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uterus and vagina. Were the levels of estrogen in the young calves treated with FSH determined?

W G MARDEN May I comment on that Follicular development in these calves was tremendous and the vaginal secretions following the FSH injections were so high that we sometimes obtained as much as 50 ml of cervical fluid This would indicate that there is quite a large amount of estrogen being produced by these follicles

IRBY M BUNDING Dr Clegg does progesterone work via the pituitary hypothalamus ovary or the duct system?

M T CLEGG I detect a note of collusion between Dr Bunding and Dr Nalbandov with respect to this question Both he and I have been interested in the role of the hypothalamus upon estrous activity in the ewe That progesterone might exert its effect upon estrous behavior by way of any one or all the structures mentioned in the question is certainly possible The evidence however is at least equivocal with regard to the participation of some of these organs in response to progesterone Thus for example as Dr Donker pointed out in his paper that progesterone might exert its effect as an anti fertility factor in humans by way of an inhibition of the pituitary ovulation stimulating hormone has been questioned by Pincus (1956 *Acta Endoc* 28 18) A direct influence of progesterone on the ovary appears unlikely since progesterone does not prevent ovulation in the rabbit following copulation when exogenous LH is given Finally the work of Hansel and his colleagues at Cornell on the cow and Everett and Sawyer on the rat indicates that progesterone may act as an ovulation stimulator in the presence of high estrogen levels and as an ovulation inhibitor in the presence of low estrogen levels by its influence upon some neural mechanism probably by way of the hypothalamus

R O BERRY Dr Donker do you feel in the light of your own observations that the low fertility of animals following progesterone treatment is due to hostility of the reproductive tract to sperm or to the production of defective eggs?

J D DONKER Considering Dr Berry s work and the work that is in the literature it appears that whatever is blocking or lowering the conception rate or the fertility is due to antagonism of the female reproductive tract to sperm It could of course be at a later stage Ulberg feels that fertilization or the meeting of the gametes is not impaired and that the lowered fertility is the result of embryonic mortality Thus the question is open to debate and certainly needs considerably more research to clarify it

IRBY M BUNDING It seems apparent from all the work that has been reported here particularly on the sheep breeding studies wherein the timing of the psychic manifestations of estrus become all important and in the attempts by many investigators to use estrogen for the job of inducing these states of psychic hyperactivity that we should give careful consideration to the question of judiciously balancing the psychic manifestations with those events which occur in the tubular portions of the reproductive tract

Now I think the time is ripe for one to look for substances which would induce the psychic activity without interfering with the tubular portions of the tract I think that herein lies the fault on which many investigators have stumbled they have given such large doses of estrogen that the muscular activity of the tract is increased to a point where the egg is shot down very quickly to an area where fertilization and or implantation is difficult or impossible

E P REINEKE If I may be permitted a comment here I believe that Mr Bunding

JOHN E NELLOR In this regard we have found that after synchronization of estrus with progesterone some heifers would come in heat again within a 4-11 day period This seemed to be associated with failure to ovulate at the synchronized estrus These heifers would continue their normal cyclic pattern whereas heifers which had ovulated at the controlled estrus would assume the pattern imposed by the new corpus luteum

WILLIAM HANSEL Are the corpora lutea formed after ovulations following progesterone treatments likely to be cystic or are they normal?

W E PETERSEN We have found that there was a tendency toward a higher than usual incidence of cystic ovaries

JOHN E NELLOR As you recall daily injections of progesterone have been necessary in the majority of the work cited in order to attain this control of estrus and ovulation Nellor and Cole (1956) found that a macrocrystalline implant of 560 mg of progesterone was capable of inhibiting estrus and ovulation 15 19 days post injection This spread in the time of estrus limited the success of a single insemination for large groups of animals For this reason we have recently attempted oral administration of progestational active compounds in an attempt to more sharply limit the post treatment estrus Ahrenhold on our campus using 17-ethinyl testosterone (a compound having about one tenth the oral effectiveness of injected progesterone in monogastric animals) could not inhibit estrus or follicular development in cycling heifers even when feeding as high as 2000 mg per day This is a tremendous amount of hormone about 5 times as much daily as necessary by subcutaneous administration to inhibit estrus and ovulation for a two week period

V W ZUERCHER Has there been any evidence of an increased incidence of pelvic fractures such as is commonly seen in the nymphomaniac following use of estrogenic hormones?

J D DONKER Yes I would say not directly due to progesterone treatment but perhaps due to after-effects of the treatment We have observed on occasion cattle thus treated to become what we consider genuine nymphomaniacs in which the bone structure was adversely affected but in the usual sense where treatment has been successful there was no such complication

W G R MARDEN Would the Panel care to speculate on

Why when young calves (under 8 weeks of age) are injected with Anterior Pituitary Extract high in FSH even though ovulation may occur estrus will be noted in only a low percentage of the calves so treated Subcutaneous pretreatment before or during FSH injection of progesterone or of stilbestrol will not noticeably increase the percentage of calves showing estrus even though superovulation may have occurred

E F GRAHAM We have on occasion stimulated ovarian growth in very young calves 6–8 weeks of age We have had almost the same results that Mr Marden has indicated We have been able to get from 100 to 140 follicles to develop on the ovaries of these very young calves The calves however did not exhibit outward symptoms of heat and I cannot answer why actual heat or symptoms of heat did not occur It was noted however that many of the follicles ovulated and several corpora lutea developed

M T CLEGG The principal effect of injections of gonadotrophins high in FSH into immature rats is to cause the development of multiple follicles which do not attain full size nor do they secrete estrogen as determined by the response of the

has brought up a very important point I was much interested in Dr Berry s statement yesterday that there was a great possibility of estrogen interfering with migration of sperm into the oviducts of these animals

A V NALBANDOV The longer I work with progesterone the less I understand how it acts For instance in chickens we can show that progesterone in certain dosages actually has gonadotrophic effect causing ovaries to enlarge Progesterone also acts as an ovulating hormone releasing substance and finally, if the dose is high enough it completely inhibits the ovary and the pituitary gland Sammelwitz reported from Illinois last year that in pigs he is capable of completely obliterating corpora lutea with high doses of progesterone to the point where you can t even see any trace of luteal tissue In the rat in contrast no amount of progesterone will induce destruction of the corpus luteum I think that we should pay a lot more attention to the dose with which we are working In regard to the sperm situation I would like to call attention to the fact that all available evidence beginning with Chang s and Blandau s work, indicates that relatively few sperm normally reach the oviduct According to some estimates not more than 100 sperm reach the oviduct in the rabbit In the cow too very few sperm literally just a few hundred reach the oviduct

W E PETERSEN Some observations that we have made in connection with fertility and superovulation may be only apropos and may further confuse the issue Nichols in his work observed that when there was evidence of a follicle developing earlier than the rest of them during superovulation it resulted in none or very few fertilized ova This of course does not at all disprove the hypothesis that faulty motility of the tract might be responsible for interfering with sperm transport But again it might also indicate that something else happens to fertilizability of the egg when one of them gets the jump on the other

JOHN E NELLOR In regard to Dr Nalbandov s comment on various levels of progesterone effecting stimulatory or inhibitory action we have noted that 560 mg of progesterone suspension if given on day –1 or day –2 of the estrous cycle will occasionally inhibit the formation of the corpus luteum In these cases the follicle developing subsequent to the progesterone inhibition does not ovulate In view of the suggestion that progesterone is necessary to initiate ovulation in other spontaneously ovulating species we have wondered whether the lack of functional luteal tissue is the endocrine deficit involved in these cases where ovulation does not occur

A point referred to earlier in Dr Donker s presentation should be clarified We did obtain a 17% conception rate but not in the usual sense of by first service Groups of heifers were treated the same day with progesterone suspension and 15 days later with gonadotrophin injection This was followed by a single insemination 48 hours later since we were interested in practical application By this method of treatment the majority of heifers came in heat 16–19 days after progesterone injection since the single insemination was not given at an optimal time It is felt that this conception rate could be improved if each animal were followed individually and inseminated 24 hours from the start of estrus

H T GIER Dr Donker you mentioned estrus repression in dogs by progesterone Could you review that work briefly? In my work with dogs I have never encountered reduced fertility at the ovulation following progesterone treatment Can you give any suggestion as to the reasons for the difference in reactions in dogs and cows?

J D DONKER The work that I referred to concerning the dog was one that Dr Dziuk gave reference to in his doctoral thesis and I cannot elaborate further Neither can I tell you very much about why the bitch appears to be different from the cow in this respect We can of course cite many many examples of species differences in

their response to various hormones I would certainly entertain any notion of Dr Gier on this matter

WILLIAM HANSEL Dr Clegg do the ova go down the oviducts faster than normal in the presence of progesterone?

M T CLEGG I would like to add something to this question of reduced fertility in the subsequent estrous cycles following progesterone withdrawal My source of information is from some of Dr Nellor s work which he originated when he was in California. This was a field experiment involving 23 Hereford heifers 500 mg of microcrystalline progesterone was injected subcutaneously without regard to the stage of the estrous cycle Fourteen days later these same animals received a second injection of 750 I U of equine gonadotrophin The following day all animals were artificially inseminated with semen from an Angus bull and then turned in with Hereford bulls Four black calves were born and 19 red calves As indicated from the calving date all the remaining animals had conceived by the first or second estrus following the treatment induced ovulation In other words a period of 310 days had passed between being placed with the bulls and the birth of the last calf All calves were dropped within a 40 day period Rather than a reduction of fertility on the contrary these results seem to suggest a beneficial effect of progesterone on subsequent conception and embryonal survival

E F GRAHAM I might refer to one experiment we conducted in which three consecutive cycles were maintained with progesterone During the third cycle six of the animals came into estrus on withdrawal of progesterone and while all animals showed visual signs of estrus only two of these on the third recycling developed a corpus luteum I think this is very important because if the administration of progesterone and its subsequent withdrawal gives us the psychic evidence of heat yet no development of a corpus luteum there is no question why we might have a very low conception rate on first service after withdrawal of progesterone

E P REINEKE I would like to bring up one point which I believe has not been mentioned directly I recall that some years ago Dr Casida of the University of Wisconsin showed that progesterone in low dosages had a stimulating effect on the ovaries of swine through some mechanism unknown at that time Where he had a very small amount of progesterone present there was a tendency toward cystic follicles Larger doses of course did suppress follicular development I believe the influence of larger doses has been largely emphasized in this discussion so far I would like to know if anyone in this group has any explanation for the apparently stimulating effect of small doses in swine

W E PETERSEN This is just incident to some work we did using different dosages with the object of ascertaining what is the optimum In going down to 25 mg there was some evidence of delaying the onset of estrus but not preventing it About 50 mg per day seems to be what is necessary to completely suppress development of estrus but at this lower level there was no evidence that estrus was stimulated I believe that 25 mg per day was the lowest level we used

J D DONKER I do not know of any work at all in cattle on this matter of ovarian stimulation and I wonder if anyone perchance has had any such observations

WILLIAM HANSEL We mentioned yesterday our earlier work in which small doses of progesterone given to cattle at the beginning of estrus did hasten ovulation Under these conditions progesterone has an ovulatory effect in the cow as it has in many other species As to an explanation of this effect, I don t think we can give a really good one at present When we first found this ovulation hastening effect of

progesterone we were inclined to think that it caused an earlier than normal release of the luteinizing hormone One might even interpret the inhibition of estrus and ovulation which occurs following larger daily doses of progesterone in the same manner In other words progesterone given daily earlier in the cycle might inhibit estrus and ovulation by shifting the pituitary from the secretion of predominantly FSH to LH at a time when no follicles have yet reached a stage at which they are able to respond to the LH by ovulation

JOHN E NELLOR In regard to the site of progesterone action we have assayed the pituitaries of progesterone treated heifers and of control heifers at a comparable stage of the estrous cycle I grant you pituitary content is probably a poor index of secretory activity but we do use this in lieu of accurate methods of measuring circu lating hormones We could find no differences at all in the pituitary gonadotropin content of progesterone treated and control heifers Although there is considerable evidence that progesterone inhibits pituitary secretion there is also good evidence that it has a direct effect on the ovary Larger amounts of gonadotropin are necessary to induce ovulation in progesterone treated heifers or in heifers during the luteal phase of the estrous cycle than during the follicular phase

WILLIAM HANSEL I certainly would agree with Dr Nellor that the assay methods for specific gonadotropins leave a great deal to be desired The problem is further complicated by the extremely transient nature of some of the anterior pituitary changes such as the rapid degranulation of P A S positive material seen in the small basophils One would have to have a pituitary from an animal killed at pre cisely the right time to show the expected changes Dr Nellor has suggested that progesterone acts directly on the ovary If progesterone has a direct effect on the ovary itself it should be able to overcome the ovulation blocking effect of atropine When both atropine and progesterone were administered to heifers at the beginning of estrus ovulation remained blocked in most cases suggesting that the effects of progesterone on ovulation are mediated through the hypothalamus and the anterior pituitary

JOHN E NELLOR It is agreed that very high levels of LH will induce ovulation in progesterone inhibited animals but can we overlook these quantitative differences?

J D DONKER I think the work of Nichols quite definitely shows that progesterone has a direct effect on the ovary or the response to the ovary as the animals which had received progesterone previous to gonadotropins only produced on the average about 30 follicles while those which did not receive any progesterone produced on the average of about 50 follicles Even though there was not a statistically significant difference it did approach the statistical level

E P REINEKE Thank you Dr Donker I know it is often the custom in a panel discussion to attempt to summarize what has been said Dr Nellor says he will summarize this for us

JOHN E NELLOR We might consider a quotation from Zawadowsky (1935) one of the earliest reported attempts in controlling estrus and ovulation in cattle He successfully induced single and multiple ovulations at all stages of the estrous cycle but encountered low fertility This was believed due to the incomplete readiness of the genital tract to secure the vitality and migration of the sperm cell It is noted that the reasons suggested by this Symposium for the low fertility encountered in hormonal controlled ovulation namely not the production of faulty ova but in the sperm participation have been available for some time

E P REINEKE I think that states the case very well Dr Nellor I want to thank the members of the panel and the audience for their participation in this very worth while discussion Thank you very much gentlemen

Problems of Infertility in Dairy Herds Phillip M Hinze Carnation Milk Farms Carnation Washington

Low-Level Antibiotic Infusions in Bovine Repeat Breeders E M Sacchi Large Animal Veterinary Research Chas Pfizer & Co Terre Haute Ind E B Smith Canton, New York and J H Tower Clarks Summit Pennsylvania

Present Status of Therapy in Animal Infertility H J Hill Department of Clinics and Surgery Colorado State University Fort Collins Colorado

Classification of Male Hypogonadism Henryk Nowakowski M D Medical University Clinic Hamburg Eppendorf Germany

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V SEMEN METABOLISM AND ARTIFICIAL INSEMINATION

ISOTOPIC STUDIES OF SEMEN METABOLISM*

R J FLIPSE
Dairy Breeding Research Center
Department of Dairy Science
The Pennsylvania State University University Park

WITH the advent of the Atomic Age radioactive isotopes of biological interest have become available on a scale not dreamed of when the early tracer experiments were conducted a quarter of a century ago Tracer studies involving radioisotopes have their advantages and disadvantages but one needs only to consult any recent issue of a scientific journal covering the field of biochemistry or physiology to see that isotopes have become an integral part of modern laboratory techniques

Artificial breeding and the Atomic Age have developed almost simultaneously While artificial breeding is an applied science it is based on fundamental studies of semen physiology and metabolism conducted over a period of many years Many of these studies have been reviewed by Mann (1954) and no attempt will be made to review the literature at this time With the advent of such highly technical procedures as the freezing of semen and the promise of eventual storage of semen at room temperature (VanDemark and Sharma 1957) the industry must place still more emphasis on the investigation of fundamental activities such as cellular metabolism The object of this paper is to demonstrate the usefulness of radioactive isotopes as tracers in approaching problems of this nature

MONOSACCHARIDE UPTAKE AND UTILIZATION

In initiating the work at this laboratory (Flipse 1954) it was shown that when spermatozoa were incubated in a buffer containing carbon 14 labeled glucose the spermatozoa acquired radioactivity which was not removed by repeated washing of the cells Thus it was established that exogenous glucose becomes associated with the sperm cell during incubation It was not determined whether the activity was located on the cell surface or within the cell Such a localization would be of interest but perhaps not of great importance

* Authorized for publication on June 3 1957 as paper No 2155 in the journal series of the Pennsylvania Agricultural Experiment Station This work was aided by grants from the National Association of Artificial Breeders the Pennsylvania State Association of Artificial Breeding Cooperatives and the Atomic Energy Commission contract No AT(30–1)–1849

viously glucose is used in preference to fructose as was reported by Vantien hoven *et al* (1952) A word of caution may be in order with regard to studies of substrate utilization Interpretation is simple when the comparison is be tween similar compounds such as glucose and fructose Other comparisons such as a sugar with a phospholipid may be complicated by such things as permeability or cofactor requirements in the simplified system

Uptake of glucose C^{14} having been established Flipse and Almquist (1955) examined the products of glucose catabolism After washed spermatozoa were incubated with glucose C^{14} the classes of products were separated as indicated in Fig 3 Carbon dioxide produced during incubation was trapped in alkali protein was precipitated with trichloroacetic acid alcohol was removed by steam distillation at pH 9 0 and volatile acids were re moved by steam distillation at pH 2 0 The residue from steam distillation was then continuously extracted with ether first at pH 9 0 then at pH

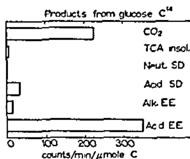


Fig 3 Distribution of radioactivity in products from the metabolism of glucose

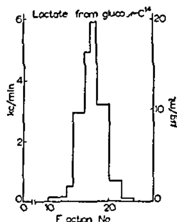


Fig 4 Elution profile of lactic acid from Dowex 1 formate column

2 0 Specific activities were much higher for CO and the acid ether extract than for the other fractions The acid ether extract was dried taken up in water and transferred to a Dowex 1 formate column The column was eluted with continuously increasing concentrations of formic acid and the fractions analyzed for radioactivity (Fig 4) after drying Although 150 of the 2 ml frac tions were collected essentially all the recovered activity was in fractions 10 to 25 Colorimetric analysis of these fractions for lactic acid by the Barker and Summerson (1941) method resulted in a curve which is superimposed on the radioactivity curve

The effects which the method of preparation of spermatozoa may have on their metabolic activity is shown in Fig 5 The usual experiment consists of a

for many enzymes function at cellular interfaces Studies of uptake by cells have obvious limitations There is first of all the problem of removing residual activity without excessive diffusion of radioactivity which has entered the cell Secondly simple measurement of uptake does not indicate the chemical form of the material taken up Thirdly a study of uptake in itself reveals little regarding metabolism of the compound It represents only the difference between labeled compounds entering the cell and those leaving the cell In its simplest form uptake is useful as an indication of metabolic activity but should not be over emphasized

Having established uptake some of the factors affecting it were studied Inactivation of spermatozoa prior to incubation generally reduced uptake indicating that in the normal cell uptake was a metabolic function rather than simple diffusion As shown in FIG 1 washing of spermatozoa prior to incubation results in increased uptake This presumably is the result of removal of energy sources present in the seminal plasma or the removal of cofactors involved in glycolysis The latter would reduce the rate of removal of C^{14} from the cell and thus appear to increase uptake

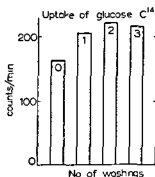


FIG 1 Effect of washing of bovine spermatozoa on the uptake of glucose C^{14}

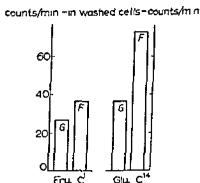


FIG 2 Uptake of glucose in preference to fructose by bovine spermatozoa G = unlabeled glucose F = unlabeled fructose

Isotopes often offer simple approaches to problems such as preferential substrate utilization *This is illustrated in* FIG 2 *Four flasks were prepared in* which the total amount of both labeled and unlabeled sugar was the same When unlabeled glucose replaced unlabeled fructose the uptake of labeled fructose was reduced and when unlabeled fructose replaced unlabeled glucose the uptake of labeled glucose was increased If a product of metabolism such as CO is measured the results are even more striking Replacing unlabeled fructose with unlabeled glucose reduced the $C^{14}O$ produced from labeled fructose by a factor of 10 replacing unlabeled glucose with unlabeled fructose increased the $C^{14}O_2$ produced from labeled glucose by a factor of six Ob

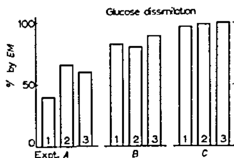


Fig 7 Evaluation of pathways of glucose degradation in three experiments by radioactivity in CO_2 (1) acetate (2) and lactate (3)

lactate agree somewhat more closely than do those with CO_2 but they are also much more difficult to recover than is CO_2 The results with each product indicate that the Embden Meyerhof pathway is predominant but they also indicate that other pathways may be present Isotopic studies should not be accepted as conclusive evidence of the existence of such pathways but should be supplemented by demonstrating enzymes involved in these pathways or isolating intermediates characteristic of these pathways

FACTORS AFFECTING PHOSPHOLIPID UPTAKE

Lardy and Phillips (1941) reported that spermatozoa readily utilize phospholipids as sources of energy Crawford Flipse and Almquist (1956) investigated the uptake of phosphorus 32 labeled phospholipids by spermatozoa Inasmuch as labeled phospholipids were not available commercially they were isolated from eggs after the hen had been fed inorganic phosphorus 32 As shown in Fig 8 the uptake by washed spermatozoa was almost three times

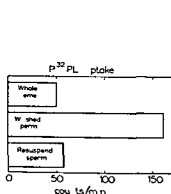


Fig 8 Comparison of uptake of P phospholipids by whole semen washed sperm and washed sperm resuspended in seminal plasma

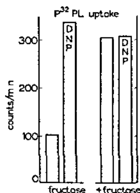


Fig 9 Effect of fructose and 2 4-dinitrophenol on the uptake of P^{32} labeled phospholipids

control of spermatozoa inactivated with acid as well as motile spermatozoa which have been freed of seminal plasma by two washings with buffer Occasionally however spermatozoa subjected to two washings show no motility when examined under the microscope With glucose C^{14} such im motile spermatozoa incorporated nearly twice as much radioactivity into CO_2 but only about 20% as much radioactivity into lactic acid as did motile spermatozoa

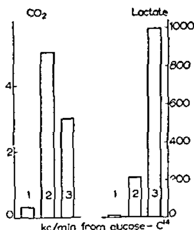


FIG 5 Production of radioactive CO_2 and lactic acid by inactivated (1) immotile (2) and motile (3) spermatozoa

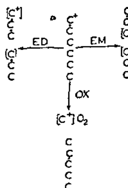


FIG 6 Schemes of glucose degradation Boxed carbons are those first appearing as CO_2

One of the attractive features of isotopes is the fact that individual atoms within the molecule can be tagged Such specific labels can be extremely useful in studying metabolic pathways (Wood 1955) Glucose 1 C^{14} was used by Flipse (1956a) in studying glycolytic pathways in spermatozoa The most widely recognized pathways of glucose catabolism are represented in FIG 6 The Embden Meyerhof process results in a symmetrical division into two 3 carbon units Carbon dioxide is not released until pyruvate enters the Krebs cycle In the Oxidative or Entner Doudoroff schemes carbon dioxide is released by splitting off carbon 1 The Embden Meyerhof process can be differentiated from the alternate pathways by comparing the production of labeled CO_2 from glucose 1 C^{14} with that from uniformly labeled glucose Other direct products of glycolysis such as lactate or acetate have some advantages over the use of CO_2 according to Blumenthal Lewis and Weinhouse (1954) We have conducted a number of experiments using glucose 1 C^{14} and uniformly labeled glucose and recovering CO_2 acetate and lactate individually or in combination The results of three experiments in which all three products were recovered are presented in FIG 7 Results with acetate and

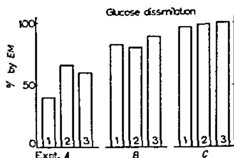


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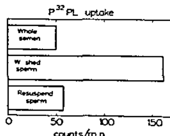


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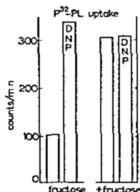


FIG 9 Effect of fructose and 2 4-dinitrophenol on the uptake of P labeled phospholipids

control of spermatozoa inactivated with acid as well as motile spermatozoa *which have* been freed of seminal plasma by two washings with buffer Occasionally however spermatozoa subjected to two washings show no motility when examined under the microscope With glucose C^{14} such im motile spermatozoa incorporated nearly twice as much radioactivity into CO_2 but only about 20% as much radioactivity into lactic acid as did motile spermatozoa

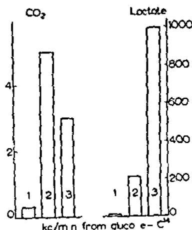


FIG 5 Production of radioactive CO_2 and lactic acid by inactivated (1) immotile (2) and motile (3) spermatozoa

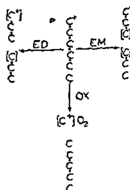


FIG 6 *Schemes of* glucose degradation Boxed carbons are those first appearing as CO_2

One of the attractive features of isotopes is the fact that individual atoms within the molecule can be tagged Such specific labels can be extremely useful in studying metabolic pathways (Wood, 1955) Glucose 1 C^{14} was used by Flipse (1956a) in studying glycolytic pathways in spermatozoa The most widely recognized pathways of glucose catabolism are represented in FIG 6 The Embden Meyerhof process results in a symmetrical division into two 3 carbon units Carbon dioxide is not released until pyruvate enters the Krebs cycle *In the* Oxidative or Entner Doudoroff schemes carbon dioxide is re leased by splitting off carbon 1 The Embden Meyerhof process can be dif ferentiated from the alternate pathways by comparing the production of labeled CO_2 from glucose 1 C^{14} with *that from uniformly labeled* glucose Other direct products of glycolysis such as lactate or acetate have some advantages over the use of CO according to Blumenthal Lewis and Wein house (1954) We have conducted a number of *experiments using* glucose 1 C^{14} and uniformly labeled glucose and recovering CO acetate and lactate indivi dually or in combination The results of three experiments in which all three products were recovered are presented in FIG 7 Results with acetate and

Glycine and glucose were compared and the glucose was found to be utilized somewhat more readily than was glycine Using identical concentrations of 0 01 M each, addition of glucose reduced CO_2 formation from glycine but the addition of glycine had little effect on CO_2 formation from glucose The 0 1 M concentration of glycine approaches the concentration effective as a semen diluent and produced some reduction in CO_2 output from glucose

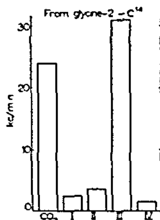


Fig 11 Distribution of radioactivity from glycine-2-C^{14} in CO_2 and cellular fractions. I = acid soluble II = phospholipid, III = nucleic acid, IV = phosphoprotein

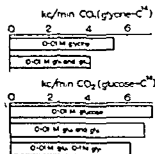


Fig 12. Oxidation of glucose and glycine in suspensions of spermatozoa in Ringer phosphate.

Having established the fact that glycine is readily metabolized by spermatozoa we have concentrated our recent efforts on the nature of this break down As shown in Fig 13 we have isolated labeled formic and glyoxylic acids from spermatozoa receiving glycine C^{14} In order to recover these compounds however, it was necessary to add a small pool of the unlabeled compound This pool served as a metabolic trap in which sufficient radioactivity accumulated to permit isolation Without the pool the labeled formate and glyoxylate were broken down as rapidly as they were formed and thus escaped detection It was noted that CO_2 production was reduced when formate or glyoxylate was added Some of this may have been due to activity trapped in formate of glyoxylate which normally would have appeared in CO_2, but it may have been due in part to inhibition glyoxylate in particular is an effective inhibitor of respiration

Figure 14 illustrates the relative amounts of radioactivity recovered in CO_2 and in formate when specifically labeled glycine was used As expected carbon 1 labeled glycine produced more $C^{14}O_2$ than did carbon 2 labeled glycine Contrary to expectations glycine 1 C^{14} also resulted in considerable labeling

that of spermatozoa in whole semen A third treatment consisted of spermatozoa resuspended in seminal plasma instead of buffer after the washing procedure This treatment was used to determine the effect of washing upon the functioning of the spermatozoa and it was found to have very little effect upon the uptake of labeled phospholipids A superficial comparison of the data presented in FIGS 1 and 8 indicates that seminal plasma has a much greater effect on the uptake of phospholipid than on the uptake of glucose Presumably this could be the result of utilization of fructose in seminal plasma in preference to phospholipids The results shown in FIG 9 would appear to contradict such an assumption for the addition of fructose to washed spermatozoa increased the P^{3} uptake Addition of an uncoupler of phosphorylation and respiration such as 2 4 dinitrophenol had the same effect as addition of fructose From this it would appear that the addition of fructose may not alter the *passage of P^{3} into the cell but delays its release* from the cell In FIG 10 the effect of *metabolic inhibitors on the uptake of phos*pholipid P^{3} is shown As expected malonate cyanide and 2 4 dinitrophenol each produced an apparent increase in uptake as a result of inhibition of the normal catabolic processes which result in the release of metabolites from the cell

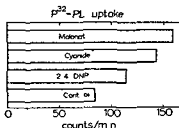


FIG 10 Effect of metabolic inhibitors on uptake of P^{32} phospholipids

METABOLISM OF GLYCINE AND RELATED COMPOUNDS

In recent months diluents consisting of glycine solutions and egg yolk or milk have been used and have resulted in an increase in the survival time and fertility of bovine spermatozoa stored at 5 C (Roy and Bishop 1954 Flipse and Almquist 1956 Flipse and Almquist 1957)

Flipse (1956b) utilized carbon 14 labeled glycine to determine the extent and nature of glycine metabolism by spermatozoa Data presented in FIG 11 represent the result of an attempt to fractionate the products of glycine metabolism Washed cells were incubated with glycine 2 C^{14} in Ringer phosphate buffer and the CO_2 produced was trapped and assayed After incubation the cells were washed to remove the medium and unused glycine and the cells were fractionated into acid soluble phospholipid nucleic acid and phosphoprotein fractions as outlined by Schneider (1945) The fractions were not purified beyond the procedure listed by Schneider but all four of the cellular fractions contained activity Semen metabolism is ordinarily considered only from its catabolic aspects and these results suggest avenues of work which have not as yet been investigated

Since considerable radioactive CO was produced from glycine we conducted the substrate preference experiments which are summarized in FIG 12

Glycine and glucose were compared and the glucose was found to be utilized somewhat more readily than was glycine Using identical concentrations of 0 01 M each addition of glucose reduced CO_2 formation from glycine but the addition of glycine had little effect on CO_2 formation from glucose The 0 1 M concentration of glycine approaches the concentration effective as a semen diluent and produced some reduction in CO_2 output from glucose

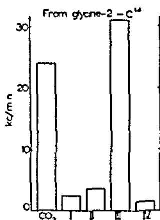


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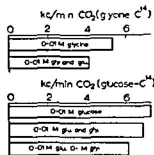


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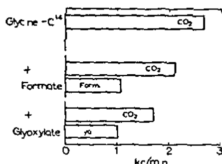


FIG 13 Production of labeled CO_2 formate and glyoxylate by bovine spermatozoa

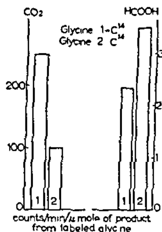


FIG 14 Production of CO_2 and formate from glycine 1-C^{14} (1) and glycine 2 C^{14} (2)

of formate A study of the relative rates of $C^{14}O_2$ production from glycine 1 C^{14} and glycine 2 C^{14} is given in FIG 15 The ratio of $C^{14}O_2$ from glycine 1 C^{14} to $C^{14}O_2$ from glycine 2 C^{14} was found to become smaller as the incubation period was lengthened from 10 to 120 min This is to be expected as a result of the carbon 1 going rather directly to CO_2 while carbon 2 is involved in intermediate steps not involving carbon 1

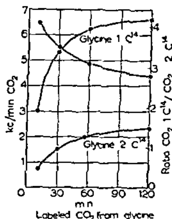


FIG 15 Rate of incorporation into CO_2 of the 1 and 2 carbons of glycine

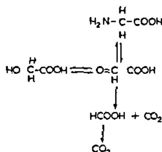


FIG 16 Scheme for the oxidation of glycine

The scheme shown in FIG 16 has been established in mammalian liver and kidney (Nakada and Weinhouse 1953) and could account for the results which we have discussed Since glycolic acid is involved in this scheme we have included it in our investigations In FIG 17 the production of $C^{14}O_2$ from

glycine and glycolate is compared Since the permeability of the cell wall to glycolic acid was not known the experiment was performed on homogenates of spermatozoa and on spermatozoa treated with versene (Koefoed Johnson and Mann 1954) in addition to washed spermatozoa Permeability did not

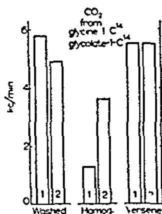


FIG 17 $C^{14}O_2$ production from glycine 1-C^{14} (1) and glycolate 1-C^{14} (2)

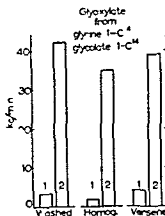


FIG 18 Glyoxylate-C^{14} formation from glycine 1-C^{14} (1) and glycolate 1-C^{14} (2)

seriously limit the degradation of glycolate for it was readily converted to $C^{14}O_2$ Figure 18 shows the production of labeled glyoxylic acid by the same preparations Glyoxylate was readily recovered following glycolate administration even without the use of a pool of unlabeled glyoxylate As was indicated earlier in the discussion a trap was necessary for the recovery of glyoxylate from glycine These results indicate that the limiting reaction in the sequence presented is the conversion of glycine to glyoxylate

The mechanism involved in the conversion of glycine to glyoxylate has not been elucidated but there are indications that transamination may be involved Tosic and Walton (1950) were unable to demonstrate the liberation of ammonia from glycine by spermatozoa In our studies glycine has failed consistently to increase the oxygen uptake above endogenous levels Perhaps the most logical transamination scheme would be one involving the amino acids *glycine and alanine and the keto acids pyruvate and glyoxylate* Such a scheme would help to explain the reduced production of lactic acid observed by Flipse and Almquist (1957) in glycine containing diluents

DISCUSSION AND SUMMARY

A discussion of all of the implications of the experiments which have been cited as examples of the use of radiotracer techniques is beyond the scope of this paper It is obvious that spermatozoa are capable of extensive metabolic

processes involving at least certain monosaccharides, phospholipids and amino acids as well as degradation products of these compounds It has been demonstrated that the metabolism of the particular compounds studied is influenced by the presence of available substrates as well as by inhibitors co factors and other additives In addition to the generally recognized catabolic activity there has been a suggestion of synthesis in these cells This in itself may alter the approach to the problem of diluent composition

Examples have been given demonstrating the use of (1) labeled compounds to determine the uptake of sugars phospholipids and glycine by spermatozoa (2) carbon 1 labeled glucose to determine the metabolic pathways of glucose (3) various tracer compounds to study preferential substrate utilization and (4) isotopes as a means of locating and characterizing metabolic products of interest

These examples by no means indicate the limits of the use of tracers in semen physiology and metabolism The possible applications in the laboratory are without number and are limited only by the imagination of the investigator This does not mean that conventional research techniques should be aban doned in favor of tracer methods tracer techniques assume their greatest potential when used in conjunction with other modern physiological and bio chemical procedures

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DISCUSSION

Wednesday July 3 1957

Afternoon Session

F X GASSNER Presiding

SEMEN METABOLISM AND ARTIFICIAL INSEMINATION

Isotopic Studies of Semen Metabolism R J Flipse Dairy Cattle Breeding Research Center Pennsylvania State University University Park

F X GASSNER Thank you Dr Flipse for a very fine paper Will you please fill out your question blanks and they will be collected as soon as you have completed them We will begin now with the discussion of this morning s papers I should like to ask the authors to come forward and sit in the front seats so they can attend the lectern Dr Hinze will you come forward first?

Discussion of Papers Given Wednesday Morning

Problems of Infertility in Dairy Herds Phillip M Hinze Carnation Milk Farms Carnation Washington

J C OSBORNE Have you seen impaired reproductive performance associated with subclinical ergotism If so would you please elaborate your experience

PHILLIP M HINZE We have no ergotism in the state of Washington that I know of therefore I have had no experience with this condition

J C OSBORNE Subclinical ergotism is considered to be a definite cause of embryonal death and abortions in cattle and other livestock in certain areas of North Carolina Abortions can be caused routinely with subclinical levels of ergot in laboratory animals and the constricting action of ergot on the vascular system of the uterus is thought to be the predisposing cause of abortion

CECIL BRANTON May I pose the following question to Dr Hinze Have you observed any relationship between conception rate or repeat breeding and level of milk production?

PHILLIP M HINZE There is evidence which suggests that high producing dairy cows have a lower conception rate than average or low producers but I would like to answer the question this way We have a family of cows at the Carnation Milk Farms called the Madcaps which is the highest producing family of cows in the world in regard to total butterfat production and this family has consistently been one of the best if not the best reproducing families in this herd To cite an example Carnation Homestead Daisy Madcap who twice broke the world s record for butter fat was bred only eight times to produce her first seven calves Other high producing cow families have experienced a poor breeding record therefore I think we should

assume that high production might influence breeding efficiency depending upon the inheritance of the individual and over all management associated with production

CECIL BRANTON Would you recommend that intrauterine infusion of antibiotics be used in treating repeat breeders ?

PHILLIP M HINZE As described in detail in the text of my paper intrauterine treatment with Combiotic and perhaps other antibiotics that have a low toxicity for spermatozoa has met with considerable success when the infusion was made during the time when estrogen dominated the cycle This means that the cow can be treated several hours before breeding or any time from ten minutes to three days following breeding Most treatments are made following breeding and before the embryo has made the descent to the uterus

CECIL BRANTON Do you have any information on adhesions caused by rupturing ovarian cysts? Particular reference is made to luteal cysts

PHILLIP M HINZE I can t give any exact figures on ovarian adhesions at this time However I recovered all of the genitalia from cattle slaughtered from our farm for several years and adhesions were found in only a very small percentage of these cows Many of them had been victims of cystic ovaries and therefore subjected to the usual therapy It was my conclusion that adhesions were associated with hemorrhage and traumatic or infectious exudates and not necessarily with the type of cyst involved Naturally the luteal cysts are more prone to trauma because of their greater resistance to manual rupture but a thin walled easily ruptured cyst may also produce a vast amount of hemorrhage Also one can t help but wonder if hemorrhage doesn t occur on occasion from spontaneous rupture of a cyst or even from the normal rupturing of a follicle because unilateral as well as bilateral ovarian adhesions have been noted in virgin heifers with no evidence of genital disease

V W ZUERCHER Do you think there may be a protein sensitivity factor adversely affecting conception in repeat breeders that have not responded to treatment show no gross pathology and manifest regular estrus? (We have noted that a fair per centage of this type animal will conceive on natural service)

PHILLIP M HINZE Regarding protein sensitization I recall three cows in our herd which absolutely would not conceive on artificial insemination when bred with diluted semen These same cows would conceive when bred with undiluted semen I bred one cow eight times in one year with diluted semen in routine insemination without conception I then bred her with undiluted semen and she conceived The following three years I bred her to routine insemination without conception for one or two breedings and then I would go to undiluted semen and she would conceive It therefore appears that protein sensitization is a factor in repeat breeders in a small percentage of cases

A M SORENSEN Did your observations show that cystic ovaries was a hereditary characteristic? Have you examined cows at slaughter for adhesions in the area of the ovary which may have been caused by manually rupturing cysts to expel the C L ?

PHILLIP M HINZE I would like to refer that question to Dr R E Erb of Washington State College who is in the audience Dr Erb had the patience to run down all the data on this cystic ovary summary and should be able to elaborate on this subject

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showing a particular characteristic have it more frequently than daughters from cows not showing the characteristic The difference in percentage of occurrence between the two groups multiplied by two yields a crude estimate of heritability The cows were classified as showing cystic tendency if they had two or more estrous cycles of fifteen days or less during the same reproductive period or had cystic follicles present on the ovary Under these conditions cystic tendency showed a heritability of approximately 0 35 Further studies involving estrus after conception and twinning rates show these characteristics occurring with cystic tendency in a non random manner Odd sex ratios have also been observed suggesting presence of a female lethal(s) Retained placenta rates are also above the herd average for the previously mentioned characteristics The possibility that cystic tendency estrus after conception twinning rate and deranged sex ratios are interrelated is currently being investigated

G W McKay Having treated a cow with cystic ovaries once how long do you wait before considering retreating?

Phillip M Hinze This of course depends a lot on each individual case but in general I give them from three to four weeks to resume a normal cycle If a cow is going to respond to treatment the vulva will usually lose its turgidity considerably ahead of the time a normal heat period is due therefore observations should be made periodically on the external genitalia to note any progress If the vulva remains turgid for two weeks or more following therapy there is a good chance that further treatment is necessary If the vulva atrophies following treatment a normal heat period will usually follow

Kenneth McEntee How frequently do you diagnose cystic corpora lutea as compared to cystic follicles? What are the clinical manifestations of cows with luteal cysts?

Phillip M Hinze Luteal cysts are definitely in the minority in so far as clinical manifestations are concerned perhaps in the neighborhood of 5% in my area Clinical symptoms of the classical luteal cyst are anestrum a slightly turgid vulva and only rarely a mucous discharge from the vagina The cysts are uniform in size between individual cows varying only slightly in size from that of a large marble to that of a black walnut They are thick walled and difficult to rupture Diagnosis can be confirmed by draining them with a hypodermic needle via the vagina

Kenneth McEntee Mr Chairman may I make a remark I realize that cystic corpora lutea are difficult to diagnose I have seen quite a few luteal cysts in experimental heifers which were slaughtered The general impression is that they are rare and that they cause anestrum However we examined a large number of bovine ovaries at a local slaughter house and found that cystic corpora lutea were approximately three times as common as cystic follicles In our experimental heifers with cystic corpora lutea we did not observe anestrum They had estrous cycles of relatively normal length but were somewhat irregular at the time of heat

Phillip M Hinze My results are based entirely on clinical evaluation and not slaughter house data I tried to bring out the fact in my paper that there are classical luteal cysts and classical follicular cysts and then there are a number of cystic ovaries that fall somewhere in between these two extremes both in physical appearance and in the manner in which they influence the estrous cycle I think Dr McEntee and I differ in our manner of interpretation as to just what a cystic corpus luteum is Slaughter house data are more accurate than clinical interpretation and I am pleased to have this information

Hermann Meyer Do you have a theory which might explain the high twinning rate in cows with cystic ovaries?

PHILLIP M HINZE My thoughts on this are strictly theory but I think that manual manipulation of the ovary will alter follicle production and when the ovary is dis turbed by manual rupture more than one follicle may mature at the subsequent heat period The theory behind ovarian massage is that it stimulates increased blood flow to the ovary and this increased blood supply may bring an abundance of gonado tropins to the ovary which would in turn mature and ovulate more than one follicle

LESTER S LARSON What was the conception rate of controls of repeat breeders where the intrauterine treated animals conception was 50% at first service?

PHILLIP M HINZE I can t recall the figures percentagewise but it took 2 2 breedings for conception on the controls as against 1 6 breedings per conception on the trea ed group

F S SCOTT When there is a high incidence of anestrum in a well-cared for herd of dairy cows—on routine rectal examination at about 2 week intervals is it better to manually express corpora lutea found in known anestrous cows or if no C L is present should estrogens and/or gonadotrophins be injected—or is it better to do nothing and let nature take its course? Would you suggest an arbitrary period post partum to initiate therapy and/or enucleation of the C L ?

PHILLIP M HINZE There does appear to be a higher incidence of anestrum among the extra well-cared for cattle especially in some herds and a high producing cow may take longer to show her first estrus after parturition than the average cow Therefore it is difficult to say just when to initiate treatment Some cows will not show a heat period for 60–80 days after calving which is apparently normal for them Hence no effort should be made to get them into heat any sooner than this If anestrum is prolonged beyond this point then therapy should be initiated

The type of yellow body that causes prolonged anestrum usually does not lend itself well to enucleation On the other hand if there is luteal tissue present estrogens such as diethylstilbestrol will usually produce a heat period to be followed by a normal cycle If there is no luteal tissue on either ovary and they remain that way stilbestrol even in large amounts will do no good and stronger estrogens such as E C P will have to be used Alternate therapy for this condition is the use of progesterone to enhance the action of stilbestrol or gonadotrophins alternated periodically with estrogens Either treatment should be given ample time to work before further treatment is given

If I find that a cow is cycling yet having silent heat periods I estimate the approxi mate time of the next estrum and inject this cow with 10–16 mg of stilbestrol This will add just enough estrogen to stimulate a heat period yet it isn t enough in the average cow to alter the cycle and conception will occur in many of these cases if bred

HARRY HERMAN How do you favor handling uterine pus cases? Do you like stilbestrol?

PHILLIP M HINZE Uterine pus cases can be classified as anything from a muco-purulent discharge to pyometra The mucopurulent discharge coming from an uterus that is grossly normal as palpated per rectum can best be treated by injecting 10–15 mg of stilbestrol I M every three or four days for about two weeks and supplemented with intrauterine injections of soluble tetracycline The pus case that has progressed to pyometra i e uterine enlargement with varying amounts of a thin foul smelling pus is treated slightly different Before antibiotics can be of much help the uterus has to be evacuated or nearly so This is accomplished by larger injections of stilbestrol up to 35 mg every three or four days or by the use of E C P

If the pyometra is of long standing the C L is probably not producing progesterone in very large amounts and 50–100 mg of progesterone may be injected to enhance the action of the estrogens After the uterus is emptied then tetracycline should be injected into the uterus

HARRY HERMAN Do you consider extreme uterine contractions specifically caused by stilbestrol harmful in forcing pus up the Fallopian tubes and causing adhesions and the end result being blocking of the tubes?

PHILLIP M HINZE I think this is entirely possible but I have nothing to base my opinion on other than suggestions from available literature

R O BERRY How large does the cavity in the corpus luteum have to be in order to be considered cystic?

PHILLIP M HINZE I was of the opinion that these small cavities in the corpus luteum were more or less normal but after hearing Dr McEntee s report any cavity may be abnormal and therefore would have to be considered cystic Clinically it would be difficult to recognize a cyst much smaller than 2 cm in diameter

F X GASSNER Thank you Dr Hinze I am sorry I cannot allow any more questions from the floor if there were any We will have to go to the next discussion Thank you very much

Discussion of Dr Sacchi s Paper

KENNETH MCENTEE What is the value of this work without controls? Since it was not established that the treated animals had endometritis and adequately con trolled experiments have not shown antibiotics to be of value in treating this class of repeat breeders it does not seem that it was justifiable to omit controls

E M SACCHI It was justifiable from our standpoint In working with antibiotics as we do we probably know more about them than the next fellow We have done work infusing uteri and we have determined the amount of antibiotic that can be recovered in the blood stream after uterine infusion It was possible in many cases to recover up to 70–80% of the antibiotic in the blood stream Now it seems to me that if we intend to treat a condition within the womb we should not use true solutions of antibiotics but just like in the case reported this morning we have prepared solutions that would finally precipitate and nebulize the uterine cavity when ad ministered and would remain *in situ* instead of going into the blood stream As to the lack of control I would like to cite the fact that other investigators (Chambers Woelffer Herrick Lindley Easley Trotter Leonard) before ourselves have avoided that If Dr McEntee can assure me of our being able to detect abnormalities and subdivide experimental animals into two homogeneous groups then I can see the possibility of keeping controls By not keeping controls we have recognized our inability to subdivide in two equal groups the animals available However I shall try to correct this report before it appears in publication

R D ANGUS Is the antibiotic used in these trials one of the presently produced forms of terramycin such as your I V intramuscular or soluble powder or is it a new formula not yet on the market?

E M SACCHI It is a new formula in the experimental stage Our reasoning be hind the formulation of this new compound was that mentioned before Present antibiotic solutions do not remain locally with them most of the antibiotic is re covered systemically The present compound was designed for local action and with compatibility for sperm in mind two things that are seldom possible with usual antibiotic solutions

Wednesday July 3 1957

Afternoon Session

H J HILL Presiding

ROUND TABLE DISCUSSION ON ARTIFICIAL INSEMINATION—FROZEN SEMEN

Problems Involved in the Use of Frozen Semen in California by S W MEAD Department of Animal Husbandry University of California Davis

COBA s Experience with the Use of Frozen Semen by RICHARD KELLOG Central Ohio Breeding Association Columbus

Rates of Semen Freezing by M H EHLERS Department of Dairy Science State College of Washington Pullman

A Review of the Present Status of Semen Lyophilization by CHARLES P STROBLE Department of Animal Production University of Wyoming Laramie

DISCUSSION

H J HILL We want to begin this panel on artificial breeding by asking the participants to discuss various phases of frozen semen work as related to artificial breeding There are two phases the practical use of frozen semen in an artificial breeding program and the technical aspects of freezing semen

The first speaker will present information on frozen semen from the practical standpoint I introduce Richard Kellogg General Manager of Central Ohio Breeders Association

RICHARD KELLOGG Shortly after the information about frozen semen came back from England we became interested in the technique because a large number of purebred breeders in Ohio were anxious to have available the select mating made possible through a frozen semen program I think this selective mating program using frozen semen accounts for the fact that over 20% of the cows we breed are registered animals

Our first technician started using frozen semen in February of 1954 gradually area by area technicians were changed over to using frozen semen until on June 14 of this year all of the technicians in our organization were on a strictly frozen semen service About 57 000 cows were inseminated with frozen semen in 1955 and almost 93 000 in 1956 Throughout this period of time the conception rates were comparable to those reported by technicians using liquid semen from the same bulls There was less than one tenth of 1% difference between the averages of the men using liquid semen and the men using frozen semen To date those men using frozen semen this year are reporting slightly higher conception rates than those that were using liquid semen

Collected semen is diluted with half the ultimate diluting material allowed to

stand for a period of 6 hr in a cold room at 41 F After this cooling period the remainder of the diluting material containing the glycerine is added drop by drop while the samples are continually stirred by the action of an oscillating table

As illustrated in Fig 1 the glass ampules are filled and sealed automatically This particular ampule filling machine (Popper) places the desired volume of diluted semen into the ampule very gently rather than squirting it in as is true of hand filling techniques The ampules then go on by three burners which complete the seal and then on into a tray

This machine will process about 2400 ampules at a time The alcohol in the freezing tray is at 41 at the beginning of the freeze Liquid CO_2 is fed into the freezing tray at a predetermined rate designed to produce the proper rate of temperature fall A small motor drives a rotary blade to assure constant mixing of the alcohol Toward the end of the freezing process the liquid CO_2 is allowed to flow into the alcohol bath rapidly thus dropping the temperature quickly The amount of CO_2 allowed to come into the alcohol is controlled by a cam which operates the valve openings A portion of the cold alcohol can be drained off warm alcohol added to the remaining in such quantity to bring the temperature back up to 41 F and the freezing process can be started again

This machine and the automatic filling and sealing machine went into operation almost simultaneously Prior to installation of these machines our average loss during the freezing process approached 27% our average loss now is about 13% We do not have any controlled experiments to support this and it may not be due to these two machines The improvement began at about that time and we cannot help but feel that the machines are an important factor in improving our freezing techniques

After the semen is frozen it is placed in our mechanical refrigeration storage bank which stores a maximum of 58 000 ampules From this unit the semen is distributed to the technicians

Our field technicians use these Meese kits to carry the semen with them at all times The boxes store about 480 ampules and are carried in the car at all times Each technician has about 45 different bulls available at all times The box uses about 8 lb of dry ice per day which is one disadvantage to this equipment Semen supplies are transported back to the district supply banks by the three field supervisors after our regular Monday morning staff meeting These district banks supply semen for from three to seven technicians depending upon the demand

H J HILL The next speaker M H Ehlers will discuss rate of temperature drop as related to the efficiency of recovery

M H EHLERS Thank you Dr Hill Members of the Symposium

We started our semen freezing work a little later than some of you and we have been trying to catch up In many ways we have taken advantage of earlier work such as the recommendations on percentage of glycerol to use in an extender However in one particular area, the rate of semen freezing published reports have been very dissimilar From a theoretical standpoint we can think of freezing rates as being in two categories On the one hand a slow cooling rate has the advantage of allowing adjustment to environment On the other hand freezing produces changes in physical state Accompanying damage may be reduced if freezing is carried out rapidly These two theories are not mutually exclusive it is probably desirable to use different rates of freezing at different temperatures Most reported freezing processes are characterized by an acceleration in cooling rate at lower temperatures

It became apparent to me in looking over the different freezing patterns that they are not easily categorized We think of rates as being slow or being fast and yet realize that these are relative terms Another difficulty in categorizing is that there is considerable variation as to the temperature or temperatures at which freezing rates are altered There is at least one report of deceleration Rates are not always linear The maintenance of a fast decline is also of course physically limited as one approaches the minimum temperature of the cooling medium

I invite your attention to the first reference in the Table Smith and Polge (1) in 1950 used baths at successively lower temperatures transferring semen ampules from one bath to the next after selected time intervals In this reference as well as in subsequent ones temperatures refer to the cooling medium surrounding the ampules For the first trial of Smith and Polge (1) ampules were in each of the eleven stages or baths for 5 min A subsequent trial was similar except that 2½ min stages were used In a third trial two additional stages somewhat slowed the cooling rate at lower ranges Differences were not pronounced but there were somewhat better sperm survivals in the last mentioned method particularly with higher glycerol levels

Polge and Lovelock (2) in 1952 described a device which brought about a cooling rate of about 1 C/min to −15 C after which a faster rate was achieved At that time the opinion among English workers apparently was that the freezing rate should be slow in the forepart of the cooling When a temperature of −15 or −20 C was reached it was permissible to accelerate the freezing rate Bruce (3) in 1953 reported work in which a decline of 1 C/min was followed to −50 C

Subsequent to this early work freezing rates may be described as divergent Some experiments have used initial cooling of a fractional degree per minute Buch *et al* (13) of Wisconsin froze at 0 33 C/min to −10 C at an accelerated rate to −15 C and then froze at an extremely rapid rate In talking to Mr Kellogg I gained the impression that the freezing pattern followed by Central Ohio Breeders Association (COBA) is somewhat similar The most recent report that I have seen from England indicates that Bruce (14) uses a rate of 0 5 C/min to −10 C followed by acceleration A somewhat intermediate example of cooling rate is that of O Dell and Almquist (9) which starts at 0 8 C with a stepwise acceleration after −10 C Miller and Van Demark (8) in 1954 reported on a comparison of 0 25 0 50 1 0 2 0 and 4 0 C/min to −20 C after which each freezing rate was doubled More favorable results were obtained with the 1 2 and 4 C/min initial rates than with the slower initial rates Direct immersion of semen ampules into −79 C cooling medium gave inferior results

Minnesota work in 1953 (6) and 1954 (7) indicated that the optimum for freezing with 10% glycerol–milk extender was a rate of 2 C/min from 5 to −30 followed by 5 C/min thereafter For 7% glycerol yolk-citrate the optimum rate was 3 C/min to −15 C and 5 C/min thereafter A Kentucky experiment by Jones *et al* (12) compared rates of 1 1 2 2 and 3 3 C/min to −34 4 C after which temperature all ampules were frozen at 4 4 C/min With milk and yolk-citrate extenders the slowest cooling rate was inferior

I will not have time to comment on the other freezing rates reported It should be mentioned however that this hasty appraisal does not give adequate attention to interactions which may exist between freezing rates and other variables of the freezing process My chief interest is to emphasize the sizeable differences in freezing rates It is really quite amazing that successful freezings can be accomplished over this wide range of rates

Let me summarize our own work with 7 5 % glycerol–milk extender Essentially we have been trying to cut the pie in a few more sections We have used rates of 1 1

2 2 and 3 3 C/min down to −40 followed by a rate of 5 C/min thereafter to −79 C The slowest rate was inferior Subsequently we used 1 1 C/min to −10 C followed by accelerated rates and *1 1 C/min to −25 C followed by acceleration* The 1 1 C/min to −10 C followed by acceleration was favorable over delayed accelera tion However in our work thus far we have not found any advantage in starting with a slow rate as compared with an initial rate of 2 C/min or faster

TABLE I

Semen Freezing Rates

Reference	Brief description (temp = C)
1 Smith A U and Polge C *Vet Rec* **62** 115 1950	Successive 5 min stages at 0 −2 −5 −8 −13 −15 −20 −40 −60 −79 successive 2½ min stages at same temperatures successive 2½ stages at same temperatures plus additional 2 min stages at −30 −50 and −70 The latter method somewhat better particularly at higher glycerol levels
2 Polge C and Lovelock J E *Vet Rec* **64** 396 1952	Freezing device requiring about 15 min to reach −15 and an additional 15 min to −60
3 Bruce W *Vet Rec* **65** 562 1953	1 /min 0 to −50
4 Polge C *Vet Rec* **65** 557 1953	1 /min 5 to −15 3 /min −15 to −79
5 Rowson L E A *Vet Rec* **65** 559 1953	2 /min 5 to −15 3 /min thereafter
6 Graham E F and Marion G B *J Dairy Sci* **36** 597 1953	Yolk–citrate optimum 3 /min 5 to −15 5 /min thereafter (7% glycerol)
7 Erickson W E Graham E F and Frederick E C *J Dairy Sci* **37** 651 1954	Heated milk optimum 2 /min 5 to −30 5 /min thereafter (10% glycerol)
8 Miller W J and Van Demark N L *J Dairy Sci* **37** 45 1954	0 25 0 5 1 0 2 0 and 4 0/min to −20 2× these rates thereafter Also direct immersion in −79 medium 1 0 2 0 and 4 0 rates better
9 O Dell W T and Almquist J O *J Dairy Sci* **37** 652 1954	0 8/min 5 to −10 1 0/min −10 to −15 1 5/min −15 to −20 2/min −20 to −35 rapidly there after
10 Mixner J P and Saroff J *J Dairy Sci* **37** 1094 1954	2 /min 5 to −15 0 7/min thereafter
11 O Dell G D and Hurst V *J Dairy Sci* **39** 1155 1956	0 8 /min 5 to −15 3 5/min to −65
12 Jones W M Perkins J R and Seath D M *J Dairy Sci* **39** 1574 1956	1 1 2 2 and 3 3 /min to −34 4 thereafter at 4 4/min 1 1/min inferior
13 Buch N C Smith V R and Tyler W J *J Dairy Sci* **39** 1712 1956	0 33 /min 5 to −10 1/min −10 to −15 rapidly thereafter
14 Bruce W III *Int Cong on Animal Reprod* 1956	0 5 /min 5 to −10 to −16 in 1 min rapidly thereafter
15 Bratton R W Flood J C Foote R H Wearden S and Dunn H O *J Dairy Sci* **40** 154 1957	0 8/min 5 to −15 4 4/min to 7−9
16 Graham E F Erickson W E and Bayley N D *J Dairy Sci* **40** 510 1957	See 6 7

In reconciling the different freezing rates it is of note that those following a slow initial drop may be characterized as having an exceedingly rapid rate starting at around −10 C Certainly the temperature at which freezing should be accelerated appears to be a critical one

In summarizing the situation as I see it we can say (1) Semen can be frozen successfully over a rather wide range of rates However it would appear that we should have a more accurately described optima than published work presently indicates I think the evidence clearly shows that it is not only possible to freeze at relatively fast rates after −10 or −15 C but that a relatively fast rate after this is requisite for best results (2) It is apparent that many trials on freezing rates have been carried out and that such information has not yet reached publication stage I thank you for your attention

H J HILL I am sure you will have some questions for Dr Ehlers in a moment. The next speaker is from the University of Wyoming where some research is being done on freeze-drying of semen Dr Charles P Stroble will discuss this phase

CHARLES P STROBLE We undertook the experimentation in freeze-drying semen in the hope of developing a method whereby stored semen could be shipped more economically than now possible with the frozen semen I must confess that our luck has been practically nil In 1945 Cole Smith and Parkes successfully freeze dried fowl semen using 1 1 dilution with Ringer solution and 20 / glycerol After freeze drying for approximately 3 hr they were able to reconstitute and observe from 30 to 50 / motility As far as I can determine in the available literature no such success has been accomplished with bovine semen

Leidl at the University of Munich reported at the International Congress for Animal Reproduction that a few motile sperm were observed after freeze-drying in his laboratory He used the egg yolk–citrate extender and subjected his semen to 3–4 days of drying He was of the opinion that the few sperm which did survive in some way were incorporated around the sides of his freezing tube and did not under go complete drying Leidl pointed out possible reasons for sperm kill One of the most important is a possible toxic effect of glycerol If the semen is dried to less than 5 / of water the glycerol content rises at that same time to approximately 50 / In the Munich laboratory researchers found that sperm could not survive in 50 / glycerol at room temperature

Sherman of the Foundation for the Study of Genetics now called the American Foundation for Biological Research obtained results quite similar to those obtained at the Munich laboratory He also suggested the possibility that many sperm were killed on reconstitution because of the non physiological method of reconstitution In other words at some time during the replacement of water the salt content became so large to the reawakened sperm as to be toxic to them

In my experiments at Laramie I have attempted to replace glycerol with high butterfat I must confess again that I have had no success I have on several trials managed to obtain a very small number of motile sperm after reconstitution

M A BROWN I wonder if Dr Stroble would briefly outline the procedure he follows in lyophilizing semen?

CHARLES P STROBLE I have used the several different diluters I started using the egg yolk-citrate and froze the semen according to the rate as described by Polge that is 1 /min down to about −15 and then at about 4 /min to −79 Tough top vials were used They have a funnel shaped top and will fit into the tube on a small lyophilizer They were put on the lyophilizer under vacuum for about 2–3 hr

I have not tried to dry any semen to complete dryness The lyophilized semen as soon as reconstituted was removed from the lyophilizer and tested microscopically I am now trying to work with high butterfat diluters to replace glycerol I haven t had any success there are only 1 2 or 3 sperm in each microscopic field but not enough to warrant any field test

H J HILL There has always been a question among many of you from various states as to the advantages of undertaking a frozen semen operation We have some Canadians in the audience who have been in the frozen semen business for two or three years on a hundred per cent basis Dr C F Hawkins of the Waterloo Cattle Breeding Association represents one of the first studs on the Continent to convert to a one hundred per cent frozen semen program

Our reasons for converting to frozen semen in Colorado were primarily those involving economics In our small operation we could serve all of the cows that we inseminate with one bull of each breed if we could use all of the semen Therefore as of October 1956 we changed to frozen semen I think Richard Kellogg represents one of the largest operations that has finally converted to one hundred per cent frozen semen

Now the question among many of the educators and extension personnel in this field is Should we convert to frozen semen or Should we not? and why? I have asked Dr S W Mead from California to sit on this panel because I know that frozen semen is not being used in California to the degree that it is being used in the Mid west Dr Mead s comments will surely lead to some questions

S W MEAD I agreed to participate only because it appeared to me that this would be an excellent opportunity for me to raise some questions that are of concern to our organizations in California although I fully appreciate that this is an unconventional approach to a panel discussion of this nature At the University of California we have been freezing semen quite regularly in a small way since early 1953 I have also tried to follow the literature since this subject first appeared Neither our own studies nor the work reported by others have answered many of the questions that are being asked by the breeding organizations in our state These questions involve the application of the research reported to the practical use of frozen semen In spite of certain advantages many of the breeding organizations are seriously questioning the advisability of converting to frozen semen With your indulgence I would like to take just a minute to review artificial breeding in California Artificial breeding as an organized business was slow in getting started It was thought to be all right for the small herds in the relatively thickly populated areas in the east and middle western states but not for California While our herds are large they are also widely separated except for the metropolitan areas around Los Angeles and San Francisco I don t know how these figures compare with other states but the technicians work ing in one co op with which I have been associated travel an average of 3800 miles per month and some of them travel nearly 7000 miles The first shipments of semen from a semen producing business were made in early 1945 At the present time there are seven organizing groups operating five are privately owned and two are co ops All are operating under contract with the PDCA I believe all of these organizations are doing a very satisfactory job of breeding cows In fact they must or go out of business since competition is extremely keen In some counties as many as four of the seven organizations are competing for business Last year these seven businesses reported 262 000 first services representing 29 / of the 900 000 cows in our state

Fluid semen has served the purpose of these organizations very well at least up to the present time None of them has used frozen semen as a part of their regular business although at least four are trying to keep up to date on frozen semen through

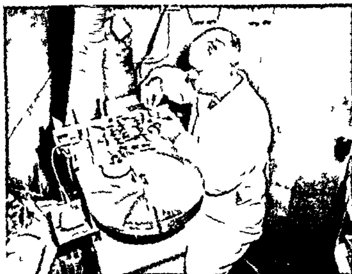


FIG 1 Automatic ampule filling and sealing machine used at COBA

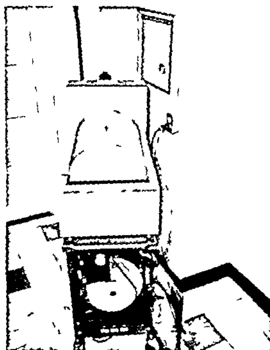


FIG 2. Automatic freezing machine (Manufactured by Frozen Semen Products Inc Route 1 Breinigsville Pa)

Fig 3 COBA s 58 000 ampule capacity storage unit (Manufactured by Cincinnati Sub Zero Inc)



[illegible] field technician s storage box (Manufactured by

freezing storing and shipping small amounts They are not ready to invest in equipment for converting over to frozen semen until they are convinced that it would be a real advantage to their clientele Certainly the use of frozen semen must either reduce costs or improve service I am not sure that it will accomplish either one under our conditions

Here are some of the questions that have been asked by the managers of some of our California breeding organizations I hope that those of you here can help us in answering these questions My first question Except for special matings is there actually a real advantage to the average dairyman of having an additional number of bulls available through frozen semen? The second question Has it been definitely established that the savings in cost through less frequent shipments will pay the initial cost and maintenance of equipment for conversion to frozen semen besides the necessary field equipment which is another large item? One co-op with which I am well acquainted has 26 technicians Of these 19 would require separate storage boxes. My third question What percentage of the good bulls now in use in the average stud would have to be culled because their semen would not freeze satisfactorily? We all appreciate the difficulty of getting good bulls After paying a big price for them and knowing that they are good bulls how many of those bulls would have to be culled because their semen would not freeze satisfactorily?

H J HILL Thank you Dr Mead I am sure you have raised many questions that have been in the minds of several people I d like to address the question relative to cost to Richard Kellogg Will you enlarge on that subject Mr Kellogg

RICHARD KELLOGG I think it is going to depend a lot upon the condition of your organization Bus shipments are the most feasible method of transportation in Ohio and yet many boxes of semen arrive a day late Our costs today for shipping liquid semen would probably run in excess of \$40 000 a year Indications are that we can put frozen semen into the field cheaper than that

H J HILL Dr Hawkins would you comment on this cost wise?

C F HAWKINS Our organization is perhaps unique compared to some in the United States in the fact that it is quite a lot smaller than most of the organizations down here Even before we started to use frozen semen we had our technicians located in central offices in the areas in which they worked We have a total of seven offices which means that we had to buy one large holding box for the central office at Waterloo and six other smaller ones (2 ft boxes) for the field offices Since that time we have added three more 5 ft³ boxes to keep up with increase in business that has taken place Our total cost was approximately \$20 000 00 which is much different than what some of you people are faced with here

We have found that in using frozen semen we have been able to obtain more business because people have a choice of sires We have found also that the owners of some large private herds carrying on a natural breeding program now employ us to freeze semen from their own bulls and to inseminate the herd artificially That not only adds dollars and cents to our business but also gives us some prestige in the neighborhood as these men are often carrying on a large operation As far as the cost of producing liquid semen and putting it in the field versus frozen semen we have not kept any accounting but I do know one must do one or the other a stud cannot carry on a dual program for any great length of time

H J HILL Thank you Dr Hawkins Another question which was asked of the panel is very pertinent Perhaps Dr Ehlers could give us some light on this Is there a real difference between bulls as to freezing and recovery from freezing?

M H EHLERS In Pullman we have not noticed any differences in freezability

patterns for bulls associated with different rates of freezing Of course as far as the freezability of different bulls certainly we have all the problems that anybody has

H J HILL Mr Kellogg is it a big problem to find bulls that will survive the freezing process?

RICHARD KELLOGG No we haven t found it to be as yet The bulls whose semen does not freeze successfully were the low conception rate bulls on liquid semen also It might be a good idea to get rid of them

H J HILL Dr Elliott could you add a comment relative to the difference in freezability of bull semen?

F I ELLIOTT Our experience has been about the same Recently we sent one bull to one of our studs which is still on liquid semen because we weren t having too much success in freezing his semen The number of such bulls is not too great and I would agree with Mr Kellogg probably the ones that don t freeze well shouldn t be in service anyway

H J HILL A number of the bulls which are presented to us in our custom freezing service are old bulls frequently physically unsound and sexually degenerating and I am sure this has an influence on their rate of recovery

S W MEAD I want to know how much advantage there is to the additional number of bulls that are made available to the average dairyman when you convert to frozen semen I know with special matings it has a distinct advantage but for the average dairyman is it a real advantage?

H J HILL Which of you gentlemen would comment on that? Dr Ehlers what would your opinion be?

M H EHLERS Being interested in the genetic aspects as all of us are I still like to feel that an average dairyman can improve his herd by selecting the bulls that he uses instead of letting all control of selection be in the hands of the organization I feel it is to his advantage to be able to select a bull to use

H J HILL Mr Kellogg do you have a comment?

RICHARD KELLOGG In a few areas about 50 / of our clientele request a particular bull In addition to that our technicians are trained to follow a program of selecting the bull that might do the best job on the particular cow being bred Certainly with frozen semen they can do a better job than with liquid semen For instance if the cow to be bred has a particular type defect then a bull is used on that cow which has done the best job of improving in his offspring that particular type defect If the technicians will do it religiously then I think we can make more improvement in the dairy cattle in the area in which we work than we have ever done before It is a big responsibility but such a program can be adapted to frozen semen American people are peculiar in that when they make up their minds as to what they want whether in our opinion it is best for them or not they are going to try to get it someplace if we can give him what he wants he is going to be happier about it The owners of some of our good grade herds are about as choosey about which bull they use as any of our purebred breeders and I am sure we are breeding more cows today because of frozen semen than we would if we didn t have it

M A BROWN I feel that where technicians help dairymen select bulls for matings to cows more emphasis should be placed on improvement of physical characteristics in progeny such as weak udders weak legs etc as well as improving milk and butter fat production We may have been over-emphasizing the production aspect at the expense of physical characteristics We still have dairymen in Texas who are not doing the best job of management and feeding so that the inheritance for milk and fat production is at a level above what we re achieving in feeding and management

This may not be true of type characteristics Loose udders or other body weaknesses may prevent some dairy animals from achieving their inherited potential for milk and fat production These defects or weaknesses can often be corrected in the cow s progeny through proper selection of the bull to which she is mated

A M SORENSEN I would like to know if we are using this technique to fulfill one of the advantages pointed out when we began to use frozen semen that is to test young bulls hold semen from such animals in a bank and then have greater use for it in the future after the bull was proven

WILLIAM HANSEL Young bulls are being tested at the New York Artificial Breeders Co operative Actually frozen semen from young bulls is not being stored except for a minimum of 25 ampules from each bull for future use in planned matings

H J HILL I think the advantages have certainly justified switching to frozen semen providing you can do it within the limits of finances you have available I concede to Dr Mead that switching to frozen semen in some instances is financially impossible It is apparent from the one year that we have been on frozen semen that after the process is known and the dairymen appreciate the choice available and the technicians become familiar with the slightly modified technique few of them would go back to liquid semen

The subject of economy of the use of semen has been raised many times The question has come up as to whether or not a bull used with fluid semen if used maximum would beget more calves than the same bull used on frozen semen because of the death rate of sperm during the freezing process I wonder if any of you who are freezing semen now still feel that frozen semen is actually more costly when we think of total number of cows bred than is liquid semen Is it going to take more or less bulls to operate a bull stud now on a frozen semen program?

RICHARD KELLOGG We are going to use less bulls particularly in some breeds However we are still going to have to maintain a certain number of bulls to provide the advantages of frozen semen such as having semen available from sires of different families lines of breeding and so on I believe we are going to be able to cut our bull numbers down by about one fourth

C F HAWKINS We have found that if anything happens to one particular sire if he goes out of service or has to be shipped to slaughter we don t have to rush right out and buy another bull to replace him We usually have plenty of frozen semen from that particular bull and the other bulls maintain the current semen production and demand

RICHARD KELLOGG Some dairymen select a particular sire for use in the herd Under the liquid semen program the use of this bull may be frequently interrupted by one or more causes which prevents delivery of semen weekly or bi weekly A frozen semen program can provide a continuous service to this dairyman because ampules are delivered from the stock supply even though the bull is temporarily out of service This is a great advantage of frozen semen over fluid

H J HILL In discussing economy as related to frozen semen with many of the bull stud managers and others it is evident that if the kill during the freeze can be kept under 30 / there is economy gained by using frozen semen Originally we questioned that When we started freezing semen here it looked as though we needed to collect more semen against the same number of cows bred but with the improve ment in freezing techniques this has changed considerably Now do any of you in the audience have any questions to be addressed to the panel? Anything relative to rates of freezing economy etc ?

J R COLLIER What is the maximum shelf life of frozen semen?

H J HILL What would you say Mr Kellogg How long have you stored semen to date?

RICHARD KELLOGG We have some semen that is about 2½ years old that still looks very good I think the length of storage time is going to depend entirely on the bull that produces it Some bulls semen will store longer than others

H J HILL I think the oldest stored semen we have is about 2½ years old and the motility is about the same as it was originally but semen of other bulls has deteriorated considerably I do not know what factor or factors are involved in semen quality that would enable us to determine how long the sample will survive storage

R O BERRY I would like to ask the panel if they know of anyone who has made a comparison of longevity of frozen sperm cells within the reproductive tract of the female as compared with stored fluid semen or with the cells from natural service

H J HILL Are there any comparisons between the longevity of sperm cells thawed after freezing and deposited in the female reproductive tract versus sperm cells stored in the liquid form?

T M LUDWICK (from Tables submitted) It has been observed that frozen semen after thawing declines in quality more rapidly than corresponding samples of liquid semen Ninety two samples of semen were divided into two portions one of which was evaluated as liquid semen and the other as frozen semen Microscopic evaluations for percent of live spermatozoa and progressive motility were made at intervals during an incubation period Each portion frozen and liquid was further divided into two different incubation temperatures 37° C and 7 C

The means of percentage of live sperm of 92 samples of semen are listed in Table I

TABLE I

Time of Incubation	Liquid semen		Frozen semen	
	37 C	7 C	37 C	7 C
0 hour	67 55	67 55	38 04	38 04
2 hours	63 00*	66 50	17.28	30 00
4 hours	57 83	65 05	5 02	27 01
6 hours	52 60*	63 50	0	20 76
8 hours	47 01	61 52	—	—

* Interpolated from original data for comparison purposes

The means of progressive motility of 92 samples of semen are listed in Table II

TABLE II

Time of Incubation	Liquid semen		Frozen semen	
	37 C	7 C	37 C	7 C
0 hour	8 49	8 49	7 12	7 12
2 hours	7 69*	8 37	2 11	5 64
4 hours	6 89	8 28	0 58	5 09
6 hours	6 09*	8 04*	0	4 04
8 hours	5 28	7 82	—	—

* Interpolated from original data for comparison purposes

A M Sorensen Mr Kellogg what percent livability are you asking for before shipping frozen semen into the field?

Richard Kellogg Most of our frozen semen would contain from 40 to 60% living sperm Some of the 40% has done equally as good a job getting cows with calves as some of the 60% however it depends too on the activity of those sperm and the concentration Microscopic study of thawed semen doesn t tell us the whole story as we all know in studying liquid semen

H J Hill Are there any other comments?

M A Brown *There is one point I would like to make concerning the seasonal* variation of bull ejaculates We find that in Texas during hot weather the quality of ejaculates from our bulls decreases to such a degree that we cannot successfully freeze the semen from those bulls If we are patient and wait until fall good ejaculates will be produced that can be frozen successfully

H J Hill Do you have any comments on that anyone on the panel? Is there a seasonal difference Mr Kellogg that is noticeable in bulls?

Richard Kellogg We have always found a slightly lower quality of semen during July and August We have found more ejaculates during that period of time that wouldn t freeze I think probably hot weather has a lot to do with it

H J Hill That is true the general lethargy the inactivity of the bull physiologically and physically reduces the quality of semen and in some instances it is not noticeable under a microscope but noticeable after the freezing process has been completed Is it possible and practical to freeze stallion semen?

M A Brown We ve had a graduate student from India who made some preliminary attempts on freezing stallion semen He was mildly successful in obtaining sperm livability after thawing frozen semen He used a buttermilk diluter with a 10% glycerol level I believe that he was successful enough to warrant further study

Fritz M Haag During the past few years we collected a rather large number of stallion semen specimens and upon taking some of these untreated samples (without the addition of glycerol or anything else) out of the dry ice box we noticed that there were some sperm coming back to life This is an observation only with no recorded data

H J Hill Is it possible to freeze boar semen?

M A Brown We have had a graduate student who has frozen boar semen The results are encouraging and this work will be continued

S W Mead *Dr Hill* may I ask a question *Has* anyone had experience in freezing ram semen? I know that someone in Australia has tried it without very much success It would be quite useful in our state

R O Berry I had one student doing this as a class project Using 7 5% glycerol and egg yolk-citrate or 7 5% glycerol and reconstituted buttermilk as a diluter in three trials he observed 12–25% sperm survival in semen that had been frozen and stored for about 3 weeks It is my opinion that ram semen will equal that of the bull in storage qualities

H J Hill Thank you very much We can conclude from this discussion and from *the literature available that frozen semen is practical It* is an economical process of inseminating dairy cattle at least, but there are still many questions unsolved namely

Why the difference between semen from different bulls and between ejaculates within bulls?

J R COLLIER What is the maximum shelf life of frozen semen?

H J HILL What would you say Mr Kellogg How long have you stored semen to date?

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What determines longevity in storage?

How can we separate those samples of semen which will not freeze and thaw satis factorily from those which do before subjecting them to the freezing process?

We still need to know the optimum rate of freezing and we have not exhausted our investigations of diluters which will work better with frozen semen than with liquid semen

If there are no other comments from the panel or from the floor I want to thank you panel members for contributing to this discussion and all of you in the audience who have taken part in it

This is the close of this Symposium I certainly express the appreciation of Colorado State University for your coming I know you have enjoyed this and that we have all gained considerable knowledge of the subject at hand I will turn this meeting back to Dr Gassner

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